ADRENAL TRANSPLANTS INTO SPLEEN AND THE INACTIVATION OF ADRENAL CORTICAL HORMONES

by GEORGE READ[1]

(From the Department of Obstetrics, University of Sydney and the Royal Prince Alfred Hospital).

(Accepted for publication 20th February, 1951.)

In the course of studies on experimental renal hypertension in rats, some aspects of the relationship of the adrenal glands to this type of hypertension have been investigated. The remaining renal artery of unilaterally nephrectomised rats with adrenal transplants in the spleen and femoral regions has been partially clamped and in both instances there have been survivors which have developed hypertension (Read, unpublished). Adrenal transplants into various tissues of animals have been described by a number of investigators (Grollman, 1936; Ingle and Higgins, 1938), but we have seen only one report of transplants into the spleen of rats and this concerned the influence of the adrenals on lymphoid tissue (Bomze and Friedman, 1950). To gain more information on this type of animal preparation, the experiments reported were designed to determine:

(1) The relative normality of rats with adrenal transplants into the spleen.

(2) The extent of inactivation, if any, of adrenal cortical hormones by direct passage through the liver.

METHODS.

Male and female albino rats weighing initially between 72 and 108 gm. were used.

Operative technique of transplants.

Both adrenals were removed at the same operation under ether anaesthesia and the capsule of one was split and the body together with the medulla expressed, leaving the capsule with adherent zona glomerulosa cells. This adrenal capsule was then pushed into a tunnel under the capsule of the spleen, prepared by making an incision into the peritoneal covering, transverse to the long axis of the spleen, and gently inserting a blunt probe for a distance of about 2 cm. Although some blood almost invariably accumulated at the end of the pocket, this did not seem to affect the success of the transplants and adhesions were rare. Depending on the type of experiment, the anatomical success of the transplants was checked by either laparotomy after four weeks or inspection after the animals had been killed.

After operation the animals were returned to their cages and put on their customary diet with 1 p.c. NaCl in tap water in the drinking bottles. This salt solution was replaced with ordinary tap water after 14 days.

---

[1] Research Fellow, King George V and Queen Mary Maternal and Infant Welfare Foundation, N.S.W.

Criteria of adrenal cortical activity.

Liver glycogen deposition.

A modification of the method of Venning, Kazmin and Bell (1946) was used. The animals were starved for 24 hours and then injected subcutaneously with a 10 p.c. glucose solution at a dosage equivalent to 200 mg./kg. of body weight. At the end of two hours, the animals were killed by a blow on the head, the livers removed and digested in hot KOH solution and the liver glycogen determined by the method of Good, Kramer and Somogyi (1933) which expresses the result as mg. glucose/100 gm. rat.

Stress.

Cold and starvation for 48 hours formed the basis of this test (Selye and Schenker, 1938). Animals were placed in cages without food and drinking water in a refrigerator at 4° C. for 48 hours and any observable differences between experimental and control animals noted.

Insulin shock.

A modification of the method of Swann and Fitzgerald (1938) was used. After a period of starvation for 18 hours, experimental and control groups were injected intraperitoneally with ordinary insulin, the dose being 2·0 units/kg. body weight. After half an hour, the injection was repeated. The onset of weakness, the time of onset and total number of convulsions, and the number of rats having convulsions were recorded.

RESULTS.

Anatomical.

The transplants took well (Fig. 1) and in no instances were accessory adrenal glands found in the original site. One animal was rejected because of adhesions between the transplant and surrounding structures. Histologically, the transplanted capsule with adhering cells regenerated in a manner roughly reproducing the normal appearance of the glands. There was an outer layer of small cells with heavily stained nuclei, and the bulk of the gland was composed of larger vacuolated cells tending to a fasciculate arrangement (Figs. 2 and 3).

Adrenal cortical activity.

Life maintenance and growth.

There were no deaths in either the experimental or control groups. Giving salt water for 14 days to the animals with adrenal transplants was probably erring on the safe side and it is possible that the transplants are functional units in a shorter time.

TABLE 1.

Gain in body weight over 10 week period.

Female	Mean body weight initial	Range	Mean body weight final	Range
Control (16 animals)	91 gm.	81–108	178 gm.	160–201
Adrenal Transplants (19 animals)	87 gm.	69–101	171 gm.	125–190
Male				
Control (8 animals)	80 gm.	75–86	260 gm.	233–286
Adrenal Transplants (8 animals)	77 gm.	72–82	215 gm.	133–250

Weight gain was reasonably uniform and consistent in both groups and all the animals remained in a healthy condition. In both the male and female transplant series there was one low weight animal (Table 1) but the weight of the remainder of the transplanted animals fell into a range similar to the controls. The rate of growth of the female animals with transplants approximated more closely to that of female controls than did similar male groups, but no experiments have been carried out to determine sex differences.

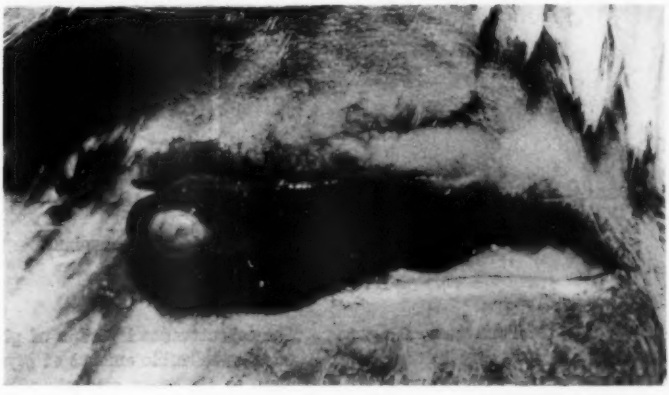

Fig. 1. Laparotomy one month after transplantation showing regeneration of the adrenal in the spleen.

Fig. 2. Low magnification micro-photograph of part of the transplanted adrenal and adjacent spleen.

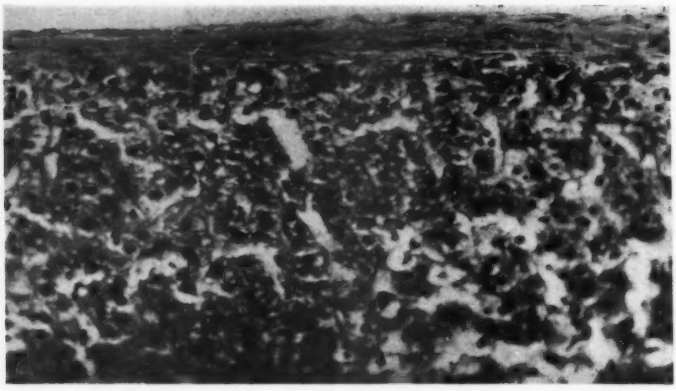

Fig. 3. High magnification of portion of the adrenal transplant.

Glycogen deposition in liver.

A 24 hour fast depleted the liver of glycogen. The subcutaneous injection of glucose in amounts already described resulted in the deposition of a considerable amount of glycogen in the liver in two hours. There was no marked difference in the mean amounts of glycogen deposited in the liver in the adrenal transplant and control groups but there was more variation in individual amounts in the former male group (Table 2).

TABLE 2.

Description of animals (6) in each group	Liver glycogen as glucose (mg./100 gm. rat)	Range
(a) Fasted female controls without injection of glucose.	1·5 mg.	0·8–2·2
(b) Fasted females with adrenal transplants, after injection of glucose.	10·2	7·2–13·7
(c) Normal controls to (b)	14·1	9·2–17·2
(d) Fasted males with adrenal transplants, after injection of glucose.	12·6	9·2–19·7
(e) Normal male controls to (d)	12·5	11·5–13·2

Stress.

Four adrenal transplanted animals and four normal controls were placed in a refrigerator for 48 hours under the conditions described. At the end of this period there was no apparent difference in the two groups. All the rats were active, alert, and healthy with no asthenia or other signs of abnormality.

Insulin shock.

In trial experiments on normal animals the amount of insulin recommended as a convulsant dose (Swann and Fitzgerald, 1938) did not appear to be likely to give any decisive information about our animals as the groups were small and the test not as sensitive as those used with mice. It was decided, therefore, to give two closely repeated intraperitoneal injections of insulin in order to show any differences in the two groups of animals. Quantitative data are represented in Table 3. It was impossible to fix a precise measure of degree of weakness; but the animals with adrenal transplants remained immobile when placed out after 35 minutes while the controls wandered about the table for a much longer period. Experimental animals and controls had been injected alternately.

TABLE 3.

Female rats	1st Convulsion after	All animals having convulsions	Deaths after 6 hours	Survivors	Total number of convulsions
Adrenal transplants (7)	40 min.	55 min.	7	Nil	88 (7 rats)
Control (7)	115 min.	Only 4 animals had convulsions	4	3 (no convulsions)	28 (4 rats)

DISCUSSION.

In our early experience with transplants we found that there was sometimes regrowth of the adrenal at its original site and, in these cases, the transplant did not grow or was small in size. It was apparent that even nicking the capsule and leaving a small fragment was sufficient to initiate the growth of a gland. With greater care and the certainty of having an intact capsule, regeneration has not occurred at the normal site. Ingle and Higgins (1938) showed that regeneration does not occur in the presence of another functional adrenal gland and this seemed to be the case with our early spleen transplants when there was regrowth at the original site. In transplantation in Addison's disease, the existence of some functioning adrenal tissue would probably militate against success and a further complication is the necessary use of heterologous transplants. However, one case of the successful grafting of an adrenal into a patient with Addison's disease has been described by Broster and Gardiner-Hill (1946).

Much information has been accumulated about the urinary excretion of products of steroid metabolism (Pincus and Thimann, 1948), but little is known of the intermediary metabolism of steroids, their action on target organs, and their inactivation. Green (1948) deduces from fluid intake studies that there is some detoxification by the liver of desoxycorticosterone implanted in the spleen of rats. Vogt (1947) summarises earlier experiments on the secretion and inactivation of adrenal cortical hormones and emphasizes the speed with which they disappear from the blood. Her experiments with eviscerated and nephrecto-

mised animals showed no detectable accumulation of the hormones in the arterial blood, several hours after operation. She concludes that neither the liver nor kidney are essential for the uptake, destruction or excretion of the circulating hormone. The present experiments are confirmatory in regard to the inactivation of the hormones by the liver. From the point of view of life maintenance, growth and stress there was little difference between the animals with adrenal transplants and the normals. The liver glycogen test is regarded as one in which responses are only obtained with corticoids having an oxygen atom attached to C_{11}. Insofar as these corticoids are concerned, the results show that there has been little or no inactivation by the liver. The mean amounts of glycogen in the transplanted and normal animals correspond, although there appears to be a variation in the individual ability of rats in both groups to store glycogen.

There was quite a difference in the two groups in the insulin shock experiments. In the adrenal transplant group, there was a much earlier and more uniform onset of weakness than in normal controls; convulsions affected all the animals and had an earlier onset, and the number of convulsions per animal was greater. At the end of a six-hour period all the animals with transplants had died whereas three of the controls, although weak, had had no convulsions; and when given their ordinary diet at the end of this period, these three rapidly became normal. The interpretation of these differences is difficult because of the absence of an adrenal medulla in the animals with transplants. Swann and Fitzgerald (1938) found an increased sensitivity to insulin in demedullated rats (transplants to ovaries) and it seems most likely that the rapid mobilization of glycogen reserves by adrenaline after insulin injection plays an important part in the time of onset, severity and number of convulsions. They found that adrenal demedullated rats were convulsed by about one-half the dose of insulin required in normal rats and are, presumably, about twice as sensitive, whereas totally adrenalectomized rats are about 24 times as sensitive. Although our experiments show a difference between animals with adrenal transplants and normals, it is much more of the order of demedullated rats. One is probably justified in assuming that it is the absence of the medulla in the transplants and not inactivation of adrenal cortical steroids which is responsible for this difference.

SUMMARY.

A method of transplanting the adrenal gland into the spleen of rats is described and the anatomical and histological result outlined. The adrenal transplanted animals were submitted to a series of tests to determine whether there was inactivation of adrenal cortical hormones by this direct passage through the liver. The degree of adrenal cortical activity was tested by the methods of life maintenance and growth, submission of stress, glycogen deposition in the liver and insulin shock.

Acknowledgments. The author gratefully acknowledges laboratory and animal house facilities placed at his disposal by Dr. A. Bolliger, Director, Gordon Craig Research Laboratory, University of Sydney. Thanks are expressed to Mr. N. Hinks for liver glycogen determinations and to Mr. D. Davidson for help in the animal work. I am indebted to Mr. S. Woodward-Smith, Department of Illustration, University of Sydney, for the photographs.

REFERENCES.

Bomze, E. J. and Friedman, N. B. (1950): Fed. Proc., 9, p. 328.
Broster, L. R. and Gardiner-Hill, H. (1946): Brit. med. J. II, p. 570.
Good, C. A., Kramer, H. and Somogyi, M. (1933): J. biol. Chem., 100, p. 495.
Green, D. M. (1948): Endocrinol., 43, p. 325.
Grollman, A. (1936): The Adrenals, p. 281. The Williams and Wilkins Co., Baltimore.
Ingle, D. J. and Higgins, G. M. (1938): Endocrinol., 22, p. 687.
Pincus, G. and Thimann, K. V. (1948): The Hormones, p. 602. Academic Press Inc., New York.
Swann, H. G. and Fitzgerald, J. W. (1938): Endocrinol., 22, p. 687.
Selye, H. and Schenker, V. (1938): Proc. Soc. exp. Biol. Med., 39, p. 518.
Venning, E. H., Kazmin, V. E. and Bell, J. C. (1946): Endocrinol., 38, p. 79.
Vogt, M. (1947): J. Endocrinol., 5, lvii.

INTERFERENCE BETWEEN INACTIVE AND ACTIVE INFLUENZA VIRUSES IN THE CHICK EMBRYO

V. THE BEHAVIOUR OF DIFFERENT STRAINS OF CHALLENGE VIRUS

by ALICK ISAACS[1] AND MARGARET EDNEY[2]

(From the Walter and Eliza Hall Institute of Medical Research, Melbourne).

(Accepted for publication 31st January, 1951.)

The non-specific nature of the interference phenomenon in animal viruses has been emphasized by a number of workers e.g. Findlay and MacCallum (1937), Lennette and Koprowski (1946), Vilches and Hirst (1947). Thus, with influenza viruses, Henle and Henle (1945) have shown that PR8 (A) virus, irradiated by ultra-violet light, interferes with the multiplication of strains of influenza A and B, and with Western equine encephalomyelitis and epidemic keratoconjunctivitis viruses, in the allantoic cavity. We have previously found that heated LEE virus interferes with the multiplication of strains of influenza A and B and of Newcastle disease and mumps viruses (Isaacs and Edney, 1950a).

In the present work, different strains of influenza virus have been tested for their interference behaviour in the allantoic cavity of the chick embryo, and clearly defined quantitative strain differences have been found. Further, the ability of the viruses tested to produce or to overcome interference has been correlated with their position in the chorio-allantoic membrane inhibitor gradient (Edney and Isaacs, 1950). In this and the following paper, the results of an investigation of strain differences are described, and their significance is discussed.

MATERIALS AND METHODS.

Virus strains.

Influenza A. WSE—Egg-adapted line of WS strain.
WSM—Mouse-lung passage line of WS strain.
NWS—Neurotropic variant of WS strain. (Stuart-Harris, 1939).

These strains have all been adapted to grow in the allantoic cavity.
They are fully described by Burnet (1951).

M—original MEL strain.
m—Variant of *M*, isolated by passage of stock MEL virus at limiting dilution.
Mr—Back-mutant of *m*, produced by growing *m* in RDE-treated eggs.
Ms—Variant of *M*, isolated by passage of *M* in homologous immune serum.

1 Working under a Special Grant from the Medical Research Council, Great Britain.

2 Francis Haley Research Officer, Department of Experimental Medicine, University of Melbourne.

M, *Mr* and *Ms* are similar to one another in the fowl red-cell gradient position and other characters; *m* differs from the other three viruses in gradient position. The MEL variants are fully described by Isaacs and Edney (1950b).

Influenza B. LEE virus.

All viruses were used in the form of infected allantoic fluid harvested 42 hours after allantoic inoculation of 11-day embryos. The embryos were incubated at 35°C. after inoculation.

Reagents.

Citrate-saline: 20 gm. sodium citrate and 9·0 gm. sodium chloride in 1,000 ml. distilled water. *Borate buffer*: Equal parts of M/20 sodium metaborate and M/5 boric acid (pH 8·5). *Calcium acetate buffer saline*: A sodium acetate-acetic acid buffer adjusted to pH 6·2 and containing 0·1 p.c. calcium chloride. *Fowl red-cells*: Thrice-washed fowl red cells made up to a 1 p.c. suspension in normal saline.

Haemagglutinin titration.

Serial two-fold dilutions of virus (0·25 ml.) were prepared in normal saline and an equal volume of 1 p.c. fowl red cells was added to each tube. The cells were allowed to settle at room temperature and readings were made by the pattern method. The amount of virus which produces partial (+) agglutination is referred to as one agglutinating dose (A.D.); haemagglutinin titres are expressed as the reciprocal of the initial dilution of virus which causes partial (+) agglutination.

RDE.

An extract of cultures of the 4Z strain of *V. cholerae* containing the receptor-destroying enzyme (RDE) was prepared as described by Burnet and Stone (1947). The RDE had a titre of 4,000 units per ml.

Fowl red cell gradient position.

A series of two-fold dilutions (4 ml.) of RDE was prepared in calcium acetate buffer saline and 0·1 ml. packed fowl cells was added to each. These were incubated at 37° C. for 30 minutes, and the action of the RDE stopped by adding sodium citrate to a final concentration of 2 per cent. The cells were washed in citrate-saline and suspended in normal saline to a final concentration of 1 per cent. Equal volumes (0·25 ml.) of virus made up to 5 A.D. and RDE-treated red cells were mixed and the cells allowed to settle at room temperature. Readings were made by observing the pattern of sedimented cells.

Preparation of heated ("indicator") viruses.

"Indicator" viruses were prepared by the methods of Stone (1949a).

The four MEL viruses and three WS viruses were diluted as follows:

Allantoic fluid—6 parts: citrate-saline—2 parts: borate buffer—1 part.

The four MEL viruses were then heated at 56°C. for 30 minutes. The indicator forms of the MEL viruses are referred to as T-*M*, T-*m*, T-*Mr* and T-*Ms*.

The three WS viruses were heated at 50°C. for 30 minutes. The indicator forms of the WS viruses are referred to as T-WSE, T-WSM, T-NWS.

Preparation of heated (interfering) virus.

LEE allantoic fluid harvested not more than seven days previously was used. It was heated for one hour at 56°C. on the day on which it was required for an interference experiment. The infectivity of each preparation was tested by inoculating 0·05 ml. of undiluted fluid into six eggs; this amount is less than the minimal interfering dose of heated LEE (Isaacs and

Edney, 1950a) and hence would not be sufficient to inhibit completely haemagglutinin production by any live virus present. All preparations in these experiments were found to be non-infective.

Determination of virus enzymic activity.

The method was based on that described by Burnet (1949). The chorio-allantoic membranes of normal 11-day embryos were ground with alundum and suspended in normal saline, 1 ml. for each membrane (Edney and Isaacs, 1950). To 1 ml. of suspension, virus was added to a final concentration of 100 A.D. per ml. with 200 units of penicillin as a bacteriostatic agent. This was incubated for 16 hours at 37° C. The suspension was then diluted 1 in 10 in normal saline and heated at 65° C. for 30 minutes in order to inactivate virus haemagglutinin. Finally, the suspension was titrated for its inhibitor content against 5 A.D. of the appropriate indicator viruses. As a control the suspension was incubated without virus. Titres in the Tables are expressed as percentages of the titre with the control suspension.

Infectivity titration.

Serial ten-fold dilutions of virus were prepared in 10 p.c. horse-serum saline. 0·05 ml. volumes were inoculated allantoically into groups of six 11- or 12- day embryos. After incubation for 42 hours at 35° C., the allantoic fluids were harvested and one drop added to 0·25 ml. of 1 p.c. fowl red cells, the presence of agglutination being taken as the criterion of infection. The 50 p.c. infectivity end-point (ID_{50}) is the dilution of virus which will infect 50 p.c. of the inoculated eggs.

Interference experiment.

Groups of six 11- or 12-day chick embryos were inoculated allantoically with heated (interfering) virus. If 0·5 ml. or greater volumes were injected, an equal volume of allantoic fluid was first withdrawn. The eggs were incubated for one hour at 37° C. and then inoculated again with 0·05 ml. of challenge virus. Controls were inoculated with the same amount of challenge virus only. The eggs were then incubated for 42 hours at 35° C. when the allantoic fluids were harvested and titrated individually for their haemagglutinin titre. The points plotted in the Figures are the geometric mean haemagglutinin titres of the groups.

The figures give the results of single representative experiments; at least two experiments were carried out with each strain.

EXPERIMENTAL.

The experiments were carried out with a single interfering agent, heated LEE, tested against seven strains of challenge virus. In the selection of challenge viruses, it was desirable to have strains which differed from one another in as few characters as possible, so that these could be related to differences in interference. For this purpose we have compared variants of single strains—four variants of MEL virus and three variants of WS virus. Although differences between MEL and WS viruses have followed the same pattern as differences among the variants, we have avoided the former type of comparison because of the extra unknown factors involved. Two types of experiment have been carried out.

(a) A constant amount of interfering virus was used, with varying inocula of the seven challenge viruses.

(b) A series of dilutions of interfering virus was used, with constant amounts of the challenge viruses.

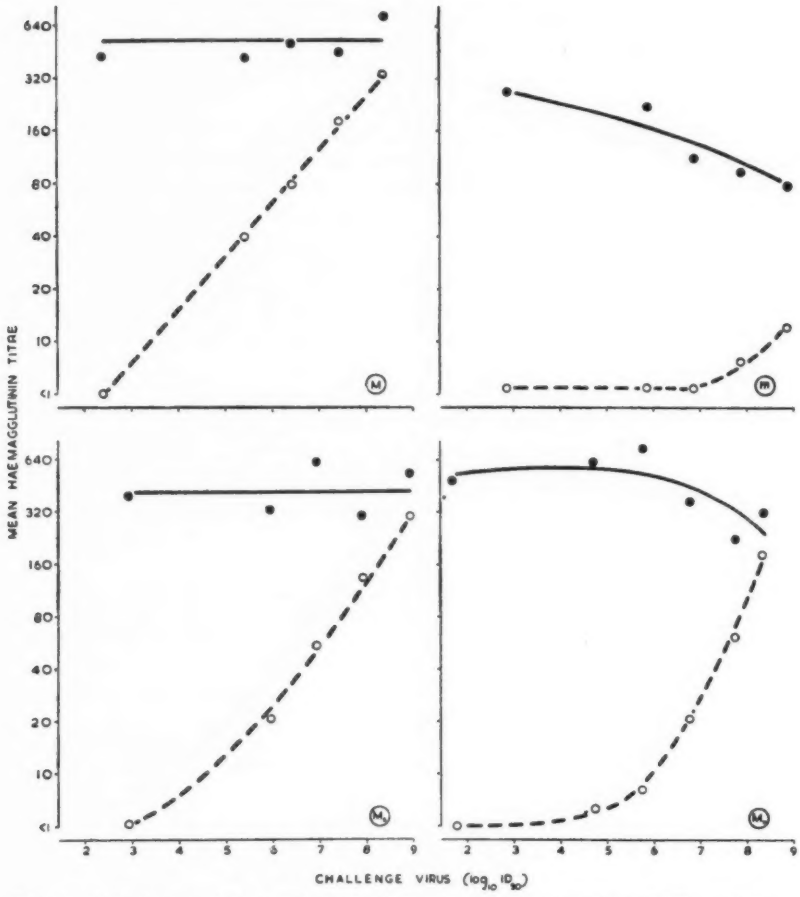

Fig. 1. Interference between heated LEE and varying inocula of MEL variants.

Challenge viruses—*M*, *m*.
Ms, *Mr*.

Interfering virus—400-800 A.D. of heated LEE.
Ordinate—geometric mean haemagglutinin titre of group of six eggs.
Abscissa—$\log_{10}$ ID_{50} of challenge virus.
Unbroken line—control curve.
Dotted line—"interference" curve.

Interference between heated LEE and varying inocula of MEL virus variants.

The four MEL variants were tested as challenge viruses against a constant amount (0·5 ml., or 400-800 A.D.) of heated LEE. In each experiment, half the embryos were injected allantoically with 0·5 ml. heated LEE and after one hour, these and the remaining control em-

bryos were injected with 0·05 ml. of challenge virus suitably diluted. The dilutions of challenge virus used were 10^{-6}, 10^{-3}, 10^{-2}, 10^{-1} and 10^{0}. After harvesting, the allantoic fluids were titrated individually for haemagglutinin, and the geometric mean haemagglutinin titres of each group were calculated. In Fig. 1 these are plotted on a logarithmic scale against the $\log_{10}$ ID_{50} of each of the challenge viruses. The upper curve, in each case, shows the titres of the control eggs; the lower curve shows the titres in the interference groups.

With all four variants, the heated LEE has produced "complete (> 99 p.c.) interference" with small inocula of challenge virus (10^2 to 10^3 ID_{50}). In the case of *M*, *Ms* and *Mr* viruses this interference was overcome when larger inocula of challenge virus were used, so that with undiluted virus (10^8 to 10^9 ID_{50}), there was practically no demonstrable interference. *m* virus showed a lower tendency to overcome interference, and with this strain there was 84 p.c. interference with $10^{8.8}$ ID_{50} of challenge virus.

Chorio-allantoic membrane inhibitor gradient of MEL viruses.

The difference in behaviour of *m* virus from the other MEL variants was interesting since tests of the position in the fowl red cell receptor gradient (Burnet, McCrea and Stone, 1946) of the four variants had shown that *M*, *Ms* and *Mr* behaved similarly, while *m* was "earlier" in the gradient than the other three viruses (Isaacs and Edney, 1950b). The possibility of similar behaviour of the MEL viruses in their enzymic activity on chorio-allantoic membrane inhibitor was considered. However, different inhibitors of virus haemagglutination are known to vary in their gradient order (Stone, 1949b) and experiments were therefore carried out to find the chorio-allantoic membrane inhibitor gradient for the MEL variants.

100 A.D. of each of the four variants were incubated for 16 hours at 37° C. with a suspension of chorio-allantoic membrane; a control sample of membrane without virus was incubated similarly. Following incubation, the residual inhibitor of each sample was titrated against the four corresponding "indicator" viruses (Methods). The results are shown in Table 1 where the inhibitory titres are expressed as percentages of the control titres.

TABLE 1.

Action of MEL viruses on inhibitor in chorio-allantoic membrane suspension.

Active virus	Inhibitory titre (p.c.)			
	T-*m*	T-*Ms*	T-*M*	T-*Mr*
m	<8	58	50	25
Ms	<8	8	<8	12
M	<8	10	<8	8
Mr	<8	<8	<8	<8

Membrane suspension incubated for 16 hours at 37° C. with active virus.

Residual inhibitor titrated against 5 A.D. of indicator viruses; titres are expressed as percentage of titre of normal membrane suspension with the same indicator.

The Table shows that *m* virus is less active enzymically on the chorio-allantoic membrane inhibitor than the other three viruses, and that inhibitor for the indicator form of *m* virus (T—*m*) is the most readily destroyed by virus enzyme action. *M, Ms* and *Mr* viruses act on membrane inhibitor to approximately the same extent in this test.

Interference between heated LEE and varying inocula of WS virus variants.

The correspondence between behaviour in interference and relative position in the chorio-allantoic membrane inhibitor gradient among the MEL variants, led to a search for a similar relationship in another system. Three variants of WS strain were available and they were tested in the same way as the MEL variants.

The results of experiments with a constant amount (0·5 ml.) of heated LEE as interfering agent tested against varying dilutions of the WS variants are shown in Fig. 2.

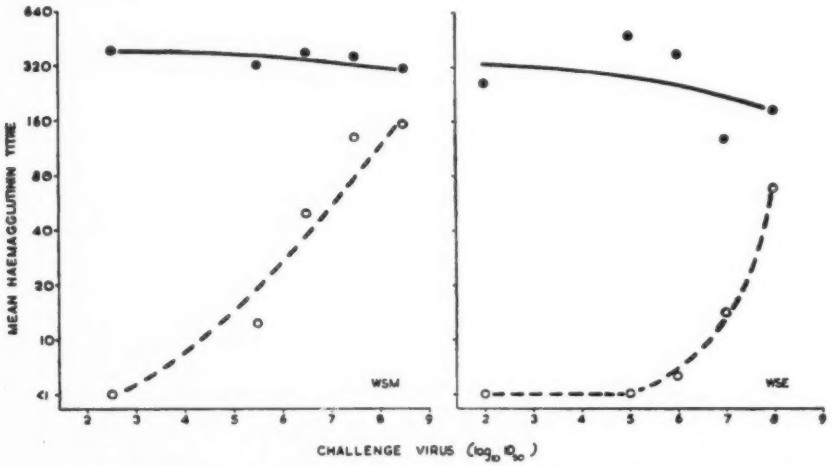

Fig. 2. Interference between heated LEE and varying inocula of WS variants.
Challenge viruses—WSM, WSE.
Interfering virus—400-800 A.D. of heated LEE.
Ordinate-geometric mean haemagglutinin titre of group of six eggs.
Abscissa—$\log_{10}$ ID_{50} of challenge virus.
Unbroken line—control curve.
Dotted line—"interference" curve.

The results of experiments of this type with NWS virus have not been graphed since:

(a) The virus showed unusually low infectivity end-points e.g. freshly harvested NWS allantoic fluid with a haemagglutinin titre of 320 had an ID_{50} of $10^{-6.2}$ only, compared with 10^{-8} to 10^{-9} for the other strains.

(b) Titrations of infectivity of NWS gave flat end-points, making any estimation of the ID_{50} difficult. Also, the haemagglutinin titres of eggs in which virus growth occurred showed a greater scatter than with any of the other strains studied.

However, the results showed that interference with NWS multiplication was greater than with the other WS strains, and there was only a very slight tendency to "overcome" interference with undiluted NWS. The greater sensitivity of NWS to interference by heated LEE can also be seen in another type of experiment, illustrated in Fig. 3.

Fig. 2 shows that WSM is more active than WSE in overcoming interference, so that the order of increasing activity for the three strains is NWS, WSE, WSM.

Chorio-allantoic membrane inhibitor gradient of WS viruses.

The three WS variants were tested for their action on chorio-allantoic membrane inhibitor. The experiments were carried out as for the MEL variants and the results are shown in Table 2. Inhibitory titres are expressed as percentages of the control titre with the same indicator virus.

TABLE 2.

Action of WS viruses on inhibitor in chorio-allantoic membrane suspension.

Active virus	Inhibitory titre (p.c.)			
	T-NWS	T-WSE	T-WSM	H-LEE
NWS	12	62	67	100
WSE	12	25	50	87
WSM	5	19	23	54

Experimental conditions as in Table 1.

The order of increasing enzymic activity of the viruses on membrane inhibitor is NWS, WSE, WSM. The order of decreasing sensitivity of the indicator form of the viruses is T-NWS, T-WSE, T-WSM. Thus the WS variants showed a correlation between order of interfering action and chorio-allantoic membrane inhibitor gradient position.

The fowl red cell gradient position of the three viruses was also investigated, using RDE-treated red cells. In this case, however, the order of the viruses found was NWS, WSM, WSE, in agreement with Burnet (1951).

Interference between varying amounts of heated LEE and MEL and WS variants.

The four MEL and three WS variants were tested as challenge viruses against varying amounts of heated LEE. A series of doubling dilutions of heated LEE was prepared and 0·5 ml. volumes injected allantoically into groups of 11-day embryos. Following incubation for one hour at 37° C. the eggs were inoculated with 0·05 ml. of challenge virus containing 10^2 to 10^3 ID_{50}. Control eggs were injected with challenge virus only.

After harvesting, the allantoic fluids were titrated individually for haemagglutinin titres and the geometric mean titres of each group were calculated. When the titres in the individual groups were expressed as percentages of the control titres and plotted against the dose of

heated LEE on logarithmic scales they gave sigmoid curves. In order to compare the different curves obtained, the percentage "scores" were transformed into probits which were then plotted against the dosage of heated LEE on a logarithmic scale. Straight lines were fitted by the method of least squares. The results are shown in Fig. 3; for convenience the percentage "scores" are shown beside the probits along the ordinate.

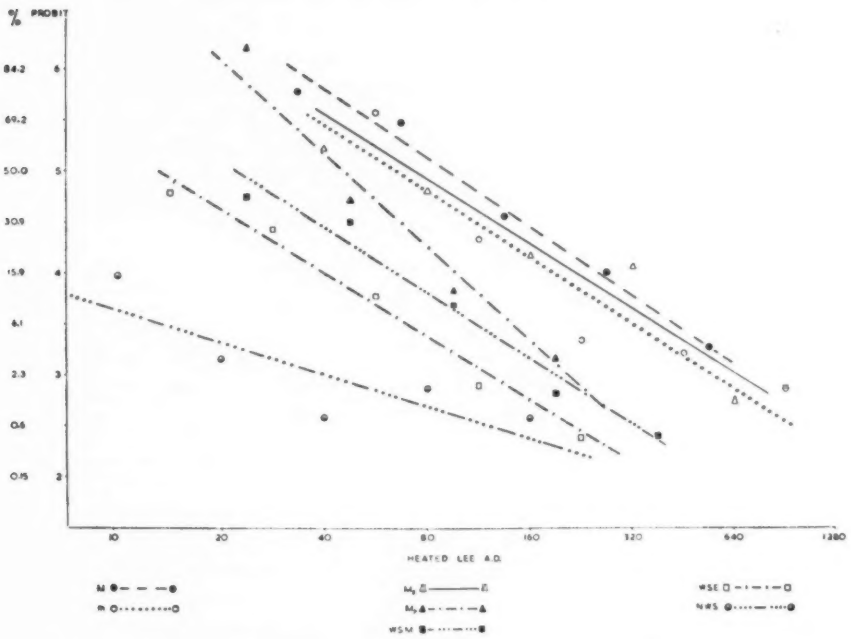

Fig. 3. Interference between varying inocula of heated LEE and seven challenge viruses.

Challenge viruses—10^2 to 10^3 ID_{50} of each strain.

Ordinate-geometric mean haemagglutinin titre of group of six eggs, expressed as percentage of control titre and plotted on a probit scale.

Abscissa—dose of interfering virus. (A.D.).

With most of the strains, the points fall quite close to the lines. NWS was the least satisfactory strain in this respect, due to the large scatter found in haemagglutinin titres of eggs inoculated with this virus. With 5 strains, the lines were roughly parallel; with the remaining strains, the lines showed different slopes.

The four MEL strains are quite close to one another; then come WSM, WSE and NWS in order of increasing sensitivity to interference.

DISCUSSION.

The experiments reported here show that with all seven strains of influenza virus investigated, interference by heated LEE caused a uniform type of response, indicated by the sigmoid type of curves obtained when the dose of heated LEE was plotted against the yield of challenge virus on a log scale. However, well-marked quantitative differences were found in the reactions of different strains of influenza virus to interference. The results shown in Fig. 3 indicate, to pick the extreme example, that 8-16 times as much heated LEE is required to produce any standard amount of interference with 10^2 to 10^3 ID_{50} of MEL (*M*) as is required to produce the same degree of interference with a similar quantity of NWS virus. Similarly, WSE virus, by this test, is approximately 4-8 times more sensitive than *M* virus to interference by heated LEE. In the first paper of this series (Isaacs and Edney, 1950a) an attempt was made to decide between two hypotheses.

(a) That complete (> 99 p.c.) interference could be produced by one particle of interfering virus per allantoic cell.

(b) That complete interference was produced by a number, x, of particles per allantoic cell, and that when $x/2$, $x/4$ etc. particles were present per cell, different degrees of partial interference resulted. At that time, it was not possible to decide between these hypotheses. However, it is now clear that, taking the minimal assumption that one particle of heated LEE per allantoic cell is required to produce complete interference with NWS virus multiplication, 8-16 times that amount of heated LEE would be required to produce complete interference with the MEL viruses. Hypothesis (a) is therefore no longer tenable.

The type of experiment shown in Figs. 1 and 2 gives information which is not given in the experiments in Fig. 3 e.g. *m* virus behaved similarly to *M* in the latter experiments and quite differently in the former. Interpretation of differences in strain behaviour is necessarily difficult in the absence of knowledge of the various factors which may be involved, such as "burst-size" of different strains (Henle, Henle and Rosenberg, 1947). For this reason, our study has been restricted to a comparison of the behaviour of variants of single strains, on the assumption that they would resemble one another more closely than strains of different origin. Among the MEL variants, *m* was found to differ greatly from *M* in its ability to "overcome" interference by heated LEE, and in chorio-allantoic membrane inhibitor gradient position. *Ms*, a variant of *M* produced by growth in homologous immune serum, resembled *M* in interference experiments and in gradient position. *Mr*, a back-mutant of *m*, has reverted to the *M* type of behaviour both in interference experiments and in chorio-allantoic membrane inhibitor gradient position, although the change in the former was not quite so complete as in the latter. Among the WS variants, the order of increasing

activity in "overcoming" interference by heated LEE was NWS, WSE, WSM; this is the same order as that of the chorio-allantoic membrane inhibitor gradient. There is thus a strong correlation in these strains between enzymic activity on chorio-allantoic membrane inhibitor (inhibitor gradient position) and ability to "overcome" interference by heated LEE.

This correlation suggests a relationship between the behaviour of a virus in interference experiments and its relative position in the chorio-allantoic membrane inhibitor gradient. Further discussion will be deferred until the following paper, when the behaviour of different strains of interfering virus is described.

SUMMARY.

Interference between heated LEE, and active strains of four MEL and three WS variants has been studied quantitatively.

400-800 A.D. of heated LEE caused complete (> 99 p.c.) interference with haemagglutinin production by 10^2 to 10^3 ID_{50} of all the strains tested. When larger inocula of the challenge viruses were used, the strains were found to differ in their ability to "overcome" interference by the same amount of heated LEE. The extent to which strains were able to overcome interference was correlated with their enzymic activity on chorio-allantoic membrane inhibitor (inhibitor gradient position).

When different amounts of heated LEE were tested against 10^2 to 10^3 ID_{50} of each of the seven challenge viruses, the strains showed large differences in the amount of heated LEE required to produce a standard degree of interference. This provides evidence that complete interference can not be accounted for by one particle of heated LEE per allantoic cell.

REFERENCES.

Burnet, F. M. (1949): Austral. J. exp. Biol., 27, p. 575.
Burnet, F. M. (1951): J. gen. Microbiol., 5, p. 46.
Burnet, F. M., McCrea, J. F. and Stone, J. D. (1946): Brit. J. exp. Path., 27, p. 228.
Burnet, F. M. and Stone, J. D. (1947): Austral. J. exp. Biol., 25, p. 227.
Edney, M. and Isaacs, A. (1950): *Ibid.*, 28, p. 603.
Findlay, G. M. and MacCallum, F. O. (1937): J. Path. Bact., 44, p. 405.
Henle, W. and Henle, G. (1945): Amer. J. med. Sci., 210, p. 362.
Henle, W., Henle, G. and Rosenberg, E. B. (1947): J. exp. Med., 86, p. 423.
Isaacs, A. and Edney, M. (1950a): Austral. J. exp. Biol., 28, p. 219.
Isaacs, A. and Edney, M. (1950b): Brit. J. exp. Path., 31, p. 209.
Lennette, E. H. and Koprowski, H. (1946): J. exp. Med., 83, p. 195.
Stone, J. D. (1949a): Austral. J. exp. Biol., 27, p. 337.
Stone, J. D. (1949b): *Ibid.*, 27, p. 557.
Stuart-Harris, C. H. (1939): Lancet, I, p. 497.
Vilches, A. and Hirst, G. K. (1947): J. Immunol., 57, p. 125.

INTERFERENCE BETWEEN INACTIVE AND ACTIVE INFLUENZA VIRUSES IN THE CHICK EMBRYO

VI. THE BEHAVIOUR OF DIFFERENT STRAINS OF INTERFERING VIRUS

by ALICK ISAACS[1] AND MARGARET EDNEY[2]

(From the Walter and Eliza Hall Institute of Medical Research, Melbourne).

(Accepted for publication 31st January, 1951.)

The relationship found between behaviour as challenge viruses in interference experiments and relative position in the chorio-allantoic membrane inhibitor gradient (Isaacs and Edney, 1950a), suggested a search for a similar relationship among different strains used as interfering agents. It was soon found that when heated PR8, MEL and LEE were compared as interfering viruses their effectiveness increased in that order; the order of these viruses in the chorio-allantoic membrane inhibitor gradient was also PR8, MEL, LEE (Edney and Isaacs, 1950). However, before any relationship is considered, it should be stressed that comparisons of this type involve at least two unknowns.

(a) It is not known how many particles of each virus compose one agglutinating dose, the standard by which interfering action has been measured. (c.f. Fulton and Dumbell, 1949).

(b) The effect of heating different strains on their ability to interfere with virus propagation is unknown. For example, Henle and Henle, (1947) showed that following irradiation of influenza viruses by ultra-violet light, various properties of PR8 and LEE were lost at different rates.

During studies on interference by heated LEE, it was observed that if the virus was treated with formaldehyde following heating, its interfering property was lost, although the virus haemagglutinin titre showed only slight changes. This observation is analogous to that of Henle and Henle (1947) on the effects of U.V. light irradiation on influenza viruses, and it suggested an experimental approach to the present problem. If the affinity of a strain of heated virus for chorio-allantoic membrane inhibitor were related to its behaviour as an interfering agent, one might expect that formaldehyde would produce a simultaneous change in interfering power and affinity for inhibitor. A series of experiments has confirmed this expectation, and the results are presented and discussed in this paper.

[1] Working under a special grant from the Medical Research Council, Great Britain.

[2] Francis Haley Research Officer, Department of Experimental Medicine, University of Melbourne.

Materials and Methods.

Most materials and methods were described in the preceding paper.

Virus strains.

In addition to those mentioned in the preceding paper, the PR8 strain of influenza virus (A) was used.

Heated virus.

All six strains used as interfering agents were heated for one hour at 56° C. Strains LEE and WSM were heated as undiluted allantoic fluid. Strains *M*, *Ms*, *m* and PR8 were diluted before heating: Allantoic fluid—6 parts: citrate saline—2 parts: borate buffer—1 part. The viruses were heated in the same way for use as "indicator" virus in these experiments.

Formolisation of virus.

Following heating, formaldehyde was added to a final concentration of 0·01–0·08 p.c., in different experiments, and the preparation incubated for 16 hours at 37° C. Generally, two or more concentrations of formaldehyde were used, and the highest concentration which did not destroy the virus haemagglutinin was used in interference tests. The final concentrations of formaldehyde used for the various strains were as follows:

LEE 0·02–0·08 p.c. : *M* 0·02–0·04 p.c. : *Ms* 0·02–0·04 p.c.
WSM 0·02–0·04 p.c. : *m* 0·01–0·02 p.c. : PR8 0·01–0·02 p.c.

With the MEL viruses and PR8, it was found that following one hour's heating at 56° C., the haemagglutinins were very sensitive to formaldehyde. These strains were therefore heated for 30 minutes at 56° C., and then formolised.

U.V. irradiation of virus.

A pool of NWS allantoic fluid was dialysed for 24 hours at 4° C., against 60 volumes of normal saline. It was then irradiated in a layer 1 mm. thick, at a distance of 20 cm., with a 3·5 amp. K.B.B. mercury arc lamp.

EXPERIMENTAL.

The plan of these experiments was as follows: Six strains of virus were heated to make them effective interfering agents. The heated viruses were then formolised. The formolised-heated and heated forms of each strain were compared for—

(a) interfering activity,
(b) affinity for chorio-allantoic membrane inhibitor.

Any test of affinity for an inhibitor of virus haemagglutination depends on the affinity of virus for the red cells used. Hence tests of the affinity of formolised-heated virus for fowl red cells were first carried out; the method used was to measure the relative positions of the viruses in the red cell receptor gradient.

Lack of interference by formolised-heated virus.

Six influenza strains were tested as interfering agents in these experiments—LEE; *M*, *Ms* and *m* variants of MEL virus; PR8; WSM. The viruses were heated and formolised as described in Methods. All interfering viruses were tested for infectivity by allantoic inocula-

tion of 0·05 ml. of undiluted virus, and all were found to be non-infective. As challenge virus, 10^2 to 10^3 ID_{50} of *M* virus were used in all experiments.

Groups of 11-day embryos were inoculated allantoically with 0·5 ml. of heated virus, and with the same volume of formolised-heated virus. After one hour's incubation at 37° C., these and control uninoculated eggs were injected with 0·05 ml. of challenge virus, and incubated at 35° C. for 42 hours. Following harvesting the allantoic fluids were titrated individually for haemagglutinin and the geometric mean haemagglutinin titres calculated for the various groups. The heated and formolised-heated viruses of each strain were tested together, but different strains were tested on different days. The results of representative experiments are shown in Table 1.

TABLE 1.

Loss of interfering property of heated virus on formolisation.

Interfering virus			Geometric mean haemagglutinin titre
Strain	A.D.	Treatment	
MEL	640	Heat	10
	560	Heat + 0·04 p.c. HCHO	400
	—	—	352
LEE	260	Heat	6
	860	Heat + 0·04 p.c. HCHO	510
	—	—	510

Results are expressed as geometric mean haemagglutinin titres of a group of six eggs. Challenge virus—100 ID_{50} of MEL (*M*) virus.

The results shown in Table 1, using LEE and MEL (*M*) viruses as interfering agents are typical of those obtained. With all six strains, heated virus caused inhibition of haemagglutinin production by MEL virus, and treatment of the heated virus by an appropriate amount of formaldehyde led to partial or complete loss of interfering power.

Position of formolised-heated virus in the fowl red cell receptor gradient.

A comparison was made between the relative positions in the fowl red cell receptor gradient of heated and formolised-heated viruses.

A series of fowl red cells treated with graded amounts of the *V. cholerae* enzyme, RDE, was tested with 5 A.D. of heated virus and formolised-heated virus. The results of a typical experiment with strain MEL (*M*) are shown in Table 2.

Table 2 shows that formolised-heated *M* is "earlier" in the fowl red cell receptor gradient than heated *M*; 0·04 p.c. formaldehyde had a greater effect than 0·02 p.c. formaldehyde.

In similar experiments with the five other strains, the same result was found i.e. in each case formolised-heated virus was "earlier" in the fowl red cell receptor gradient than the corresponding heated virus.

Progressive treatment of red cells with RDE is known to reduce the power of these to adsorb influenza viruses (Burnet, McCrea and Stone, 1946). These results suggested that formolised-heated virus had a lower affinity for red cells than heated virus of the same strain. Experiments were therefore carried out to see if formolised-heated virus also showed a lower affinity for chorio-allantoic membrane inhibitor than the corresponding heated virus.

TABLE 2.

Relative position of heated and formolised-heated MEL in the fowl cell receptor gradient.

Virus	RDE dilution						
	50	100	200	400	800	1,600	3,200
Heated MEL	—	++	++	++	++	++	++
Heated formolised MEL (0·02 p.c.)		—	++	++	++	++	++
Heated formolised MEL (0·04 p.c.)		—	—	+	++	++	++

The signs indicate the degree of agglutination of red cells treated with each dilution of RDE. 5 A.D. of each virus were used.

Affinity of formolised-heated virus for chorio-allantoic membrane inhibitor.

The determination of the affinity of a virus for an inhibitor is necessarily indirect at present. The methods used measure the distribution of virus between inhibitor and red cells and have, therefore, a serious disadvantage. Since they measure distribution, the results are dependent on the affinity of virus for red cells and do not give any quantitative indication of the extent of increase or decrease in affinity for inhibitor.

In the previous section, it was shown that formolised-heated virus of six strains had a lower affinity for red cells than the corresponding heated virus. It is therefore difficult to demonstrate experimentally a loss of affinity of heated virus for membrane inhibitor on formolisation, unless there is a proportionately greater loss of affinity for inhibitor than for red cells. This would be shown by a lower inhibitory titre of chorio-allantoic membrane suspension for formolised-heated virus than for the corresponding heated virus. If the inhibitory titre against a virus remained constant following formolisation, it would suggest an equal loss of affinity for inhibitor and for red cells.

This was tested experimentally as follows: Inhibitor titrations were carried out with chorio-allantoic membrane suspension against 5 A.D. of heated and formolised-heated virus. The six strains used as interfering agents were investigated and representative results are shown in Table 3.

TABLE 3.

Inhibition by chorio-allantoic membrane suspension of haemagglutination by heated and formolised-heated viruses.

Indicator virus	Virus strain					
	M	*Ms*	*m*	PR8	LEE	WSM
Heated virus	960	640	320	320	640	240
Formolised-heated virus	320	320	140	160	150	240

The Table shows the inhibitory titres obtained with 5 A.D. of heated and formolised-heated virus of the strains shown.

Table 3 shows that with 5 out of 6 strains chorio-allantoic membrane suspension gave a lower inhibitory titre with formolised-heated virus than with the corresponding heated virus. With strain WSM it gave the same titre.

This suggests that heated virus when formolised, had a reduced affinity for chorio-allantoic membrane inhibitor. With 5 strains, the reduction in affinity for inhibitor was proportionately greater than the reduction in affinity for red cells.

Interference between formolised-heated LEE and active NWS and MEL viruses.

In the preceding paper, it was shown that 10^2 to 10^3 ID_{50} of NWS virus was 8-16 times more sensitive than MEL virus to interference by heated LEE.

It was interesting to see whether a similar ratio would be found with formolised-heated LEE as interfering agent.

Formolised-heated LEE was tested as interfering agent against 10^2 to 10^3 ID_{50} of NWS and MEL. Two different concentrations of formaldehyde were used. As controls, groups of eggs were inoculated with heated LEE followed by NWS and MEL; and with NWS and MEL alone. The results of a representative experiment are shown in Table 4.

TABLE 4.

Interferenee between formolised-heated LEE and active MEL and NWS viruses.

Interfering virus			Geometric mean haemagglutinin titre	
			Challenge virus	
Strain	A.D.	Treatment	MEL	NWS
LEE	640	Heat	3	0
	1,600	Heat + 0·02 p.c. HCHO	32	0
	1,600	Heat + 0·04 p.c. HCHO	128	0
	—	—	304	152

Results are expressed as geometric mean haemagglutinin titres of a group of six eggs. 100 ID_{50} of each challenge virus used.

With heated LEE (treated with 0·02 and 0·04 p.c. formaldehyde) as interfering agent, the yields of MEL virus were 10 p.c. and 42 p.c. of the control, while the yields of NWS virus in the same experiment were less than 1 p.c. in each case.

Interference between irradiated NWS virus and active NWS and WSM.

If the action of an interfering virus were related to its position in the chorio-allantoic membrane inhibitor gradient, NWS virus, which is "earliest" in the gradient of the viruses tested, should be a weak interfering agent. Owing to the sensitivity of NWS virus haemagglutinin to heat (Burnet, 1950), irradiation by U.V. light was used to inactivate the virus.

Two experiments were carried out. In the first, NWS virus was dialysed and then irradiated for two minutes; this was insufficient to cause complete loss of infectivity, one out of six eggs inoculated with 0·05 ml. of undiluted virus showing haemagglutinin production. In the second experiment, the virus was irradiated for four minutes and showed complete loss of infectivity. Irradiation of NWS virus did not alter its haemagglutinin titre. In each experiment 1·0 ml. of irradiated virus was tested as interfering agent against 10^2 to 10^3 ID_{50} of NWS and WSM challenge viruses. The results of the first experiment are shown in Table 5.

TABLE 5.

Interference between irradiated NWS and active NWS and WSM viruses.

Interfering virus	Geometric mean haemagglutinin titre	
	Challenge virus	
	NWS	WSM
640 A.D. irradiated NWS	1	112
Nil	80	608

Results are expressed as geometric mean haemagglutinin titres of a group of six eggs.

100 ID_{50} of each challenge virus used.

Table 5 shows that 640 A.D. of irradiated NWS caused considerable inhibition of multiplication of NWS virus (yield of 1·25 p.c. of the control titre), but less inhibition of WSM multiplication (18·4 p.c. of the control titre). A similar result was found in the second experiment when 300 A.D. of irradiated NWS was used as interfering agent. Examination of Fig. 3 of the preceding paper shows that 60 A.D. of heated LEE produced roughly the same degree of inhibition of multiplication of NWS and WSM as did 300–640 A.D. of irradiated NWS. It thus appears that, in terms of agglutinating doses, irradiated NWS is 5–10 times less effective as an interfering agent than is heated LEE.

DISCUSSION.

In the previous paper it was shown that quantitative differences existed among 7 strains of influenza virus in their behaviour as challenge viruses in interference experiments. NWS virus was found to be the most sensitive of the viruses tested, to interference by heated LEE. It has now been found that the interfering power of 6 strains of heated influenza virus was greatly reduced on formolisation. With formolised-heated LEE as interfering agent, however, NWS virus was again found to be much more sensitive to interference than MEL virus. The extent of the reduction in interfering power *on formolisation* can be measured by reading, from Fig. 3 of the preceding paper, the amount of heated LEE which would produce the same degree of interference. Thus 640 A.D. of heated LEE, after treatment with 0·04 p.c. formaldehyde (Table 4) caused

roughly the same interference with MEL (*M*) and NWS viruses as did 80 A.D. of heated LEE. (The haemagglutinin titre of heated LEE was increased approximately two-fold by 0·02-0·04 p.c., formaldehyde treatment, but this fact was not included in the calculation). On the basis of agglutinating doses therefore, 0·04 p.c. formaldehyde caused an eight-fold reduction in the interfering power of heated LEE, in this experiment. A similar type of comparison has shown that NWS virus, irradiated by U.V. light, is 5-10 times less effective than heated LEE as an interfering agent. It has also been found that heated virus of all 6 strains when formolised showed a loss of affinity for the red cell surface and for chorio-allantoic membrane inhibitor. The main difficulty in attempting any correlation between the loss of interfering power and loss of affinity for inhibitor on formolisation, is the lack of quantitative information on the extent of loss of affinity for membrane inhibitor. From an examination of the effects of formaldehyde on different strains, it seems clear that there is no simple linear relationship between loss of affinity for membrane inhibitor and loss of interfering power. However, it can be stated qualitatively that treatment with formaldehyde has produced a simultaneous reduction in interfering power and in affinity for chorio-allantoic membrane inhibitor, of six strains of heated influenza virus.

These observations, taken with those in the preceding paper, point to a relationship between interference and affinity for chorio-allantoic membrane inhibitor. In an attempt to clarify this relationship it may be worthwhile summarizing some of the relevant information obtained with the present experimental system on the interference phenomenon in influenza viruses.

(1) With a *minimal interfering* dose of heated, enzymically inactive virus, interference is intracellular (Isaacs and Edney, 1950b).

(2) The most likely explanation of interference is that it represents a competition between inactive and active virus for cellular constituents required in virus multiplication. This hypothesis was first proposed for interference in bacteriophages by Delbrück and Luria (1942), and has been supported for interference in influenza viruses by Ginsberg and Horsfall (1949), Henle (1950), and Isaacs and Edney (1950b). It was shown in the preceding paper that the extent of interference with seven strains of challenge was a function of the amount of interfering agent; also with each of the seven strains, within the experimental limits chosen for observation, the degree of interference was a function of the amount of challenge virus used. This is direct experimental evidence in support of the "competition" hypothesis.

(3) The chorio-allantoic membrane contains an intracellular inhibitor of virus haemagglutination, which disappears during virus multiplication in the membrane (Edney and Isaacs, 1950).

(4) Among seven strains of influenza virus, those most active enzymically on chorio-allantoic membrane inhibitor were most active in "over-coming" inter-

ference by heated LEE. A similar relationship was found in interfering viruses i.e. diminished interfering power was associated with loss of affinity for chorio-allantoic membrane inhibitor.

The conclusion which these results suggest is that interference in these experiments is due, at least in part, to competition by inactive virus with active virus for intracellular chorio-allantoic membrane inhibitor. Thus NWS, which has a lower affinity for chorio-allantoic membrane inhibitor than any of the other strains studied, is also more sensitive to interference, and is a weaker interfering agent, than the other viruses. This conclusion necessarily implies that chorio-allantoic membrane inhibitor plays some part in influenza virus multiplication, a suggestion for which there is at present no direct evidence.

It should be emphasized that the conclusions reached apply only to the system studied, interference between heated and active virus in the allantoic cavity of the chick embryo. Interference between two active viruses, and other interference phenomena may have entirely different or additional mechanisms. The present experiments may, however, focus attention on the possible functions of chorio-allantoic membrane inhibitor in influenza virus multiplication.

SUMMARY.

Six strains of heated influenza virus, when formolised, showed a reduction in interfering power.

Formolised-heated virus was found to have a lower affinity for red cells than heated virus of the corresponding strain.

Formolised-heated virus was found to have a lower affinity for chorio-allantoic membrane inhibitor than heated virus of the corresponding strain.

It is suggested that interference by heated influenza virus is due to competition with active virus for intracellular chorio-allantoic membrane inhibitor.

REFERENCES.

Burnet, F. M. (1951): J. gen. Microbiol., 5, p. 46.
Burnet, F. M., McCrea, J. F. and Stone, J. D. (1946): Brit. J. exp. Path., 27, p. 228.
Delbrück, M. and Luria, S. E. (1942): Arch. Biochem., 1, p. 111.
Edney, M. and Isaacs, A. (1950): Austral. J. exp. Biol., 28, p. 603.
Fulton, F. and Dumbell, K. R. (1949): J. gen. Microbiol., 3, p. 97.
Ginsberg, H. S. and Horsfall, F. L. Jr. (1949): J. exp. Med., 89, p. 37.
Henle, W. and Henle, G. (1947): *Ibid.*, 85, p. 347.
Henle, W. (1950): J. Immunol., 64, p. 203.
Isaacs, A. and Edney, M. (1950a): Austral. J. exp. Biol., 29, p. 169.
Isaacs, A. and Edney, M. (1950b): *Ibid.*, 28, p. 231.

GLYCOLYSIS IN *PARAMECIUM CAUDATUM*

by MARGARET GEDDES AND G. F. HUMPHREY[1]

(From the Department of Biochemistry, University of Sydney).

(Accepted for publication 11th January, 1951.)

The ciliate protozoan, *Paramecium caudatum*, is widely distributed in nature, occurring in aerobic environments such as the surface layers of ponds and in relatively anaerobic conditions such as the paunch of ruminant animals (von Brand, 1946). Under these widely differing conditions, growth, movement and other energy-requiring processes are carried out. From the biochemical point of view, respiration, glycolysis and mixed fermentation reactions can each supply energy for carrying out these activities. Many investigations have been made on parasitic protozoa with regard to the occurrence and type of the biochemical mechanisms which are exhibited by these organisms (von Brand, 1946; Harvey, 1949). This work has shown that O_2 is consumed and CO_2 liberated by respiratory processes often similar to those found in higher animals; greater differences are found with regard to glycolysis and fermentation. The end-products of carbohydrate breakdown comprise CO_2, fatty acid and other organic acids.

In the case of *P. caudatum*, carbohydrate reserves are built up and broken down depending on the food available and the environmental conditions; thus, under anaerobic conditions carbohydrate reserves are diminished (von Brand, 1946). Cunningham and Kirk (1941) have shown that incubation of *P. caudatum* with added starch or glucose leads to an increase in the titratable acidity of the medium.

In continuation of the work already carried out with *P. caudatum* (Humphrey and Humphrey, 1948) and the investigations on invertebrate glycolysis (Humphrey, 1949), this present study of the glycolytic mechanism in *P. caudatum* was made.

EXPERIMENTAL.

Material.

Our thanks are due to Professor Tiegs, Department of Zoology, University of Melbourne, for supplying a culture of *P. caudatum*. A clone was established and grown in the manner previously described (Humphrey and Humphrey, 1948).

[1] This investigation was supported by apparatus grants from the Research Committee and Cancer Research Committee, University of Sydney.

Nicotinamide was obtained from British Drug Houses and Wyeth Inc. Crystalline alcohol dehydrogenase was prepared according to the method of Racker (1950). Adenylic acid was prepared from adenosinetriphosphate (ATP) by the method of Kerr (1941); analysis for N, P and molecular extinction coefficient at 260 mμ showed it to be 98 p.c. pure. Other materials were obtained as previously stated (Humphrey, 1950).

Methods.

All spectrophotometric data were obtained with a Beckman, model DU spectrophotometer. Diphosphopyridine nucleotide (DPN) was estimated by the method of Le Page (1947), using hydrosulphite reduction, and confirmed by the method of Racker (1950) with alcohol dehydrogenase. The samples of DPN which were used in the metabolic experiments assayed 58 to 69 p.c. purity.

In the previous series of experiments with *P. caudatum* in which the O_2 consumption of homogenates was determined (Humphrey and Humphrey, 1948), a Cartesian diver ultramicrorespirometer was used; because of the relatively small number of organisms needed in that investigation, the protozoa were separated from contaminating bacteria and moulds by a process of electro-migration. In the present work several million cells were needed each day and a different method of preparing the suspension was used. The culture was grown in 2 l. volumes in 4 l. Haffkine flasks; after filtering off the mould through cotton wool the suspension was centrifuged at 0° C. for 10 minutes at 2,500 r.p.m. The supernatant fluid was quickly sucked off before the organisms (slowed down by the low temperature) could rise to the surface. The concentrated suspension was then washed with 1:100 Osterhout solution as previously described. Bacterial contamination in the final experimental suspension was estimated by observing colony formation on agar plates; on the average about 1 bacterium was associated with each protozoan cell (Stockdale, unpublished).

Extracts of the protozoa were made by freezing moderately concentrated suspensions (10^5/ml. in 1:100 Osterhout solution) in an alcohol bath at —10° C. After about 15 minutes the suspension was thawed at 28° C., the process was then repeated and the cell residues centrifuged off for 5 minutes at 2,500 r.p.m. Generally 2 cycles of freezing and thawing proved sufficient for complete disintegration but sometimes 3 were used.

Reaction mixtures were incubated for 2 hours at 28° C. in the presence of 0·01 M Sörenson phosphate buffer (pH = 7·3) and the techniques used for chemical analysis, incubation etc., were the same as those employed in previous investigations (Humphrey, 1950). The concentration of protozoa (or their equivalent) in the final experimental mixture was 1–7 × 10^5 cells/ml.

Adenyl compounds were estimated in extracts by incubating with rat muscle brei (Conway and Cooke, 1939) and determining the NH_3 liberated.

Results.

Products of glycolysis.

When cells or extracts were incubated in the presence of various substrates lactic acid was formed; pyruvic acid could not be detected under any conditions. The amount of acid produced was expressed as μl. lactic acid/hour/10^4 cells (Z_L), mg. dry weight (Q_L) or mg. N [$Q_L(N)$]; the values of these quotients for anaerobic acid production from glucose in the presence of suitable activators were of the following order: (whole cells) $Z_L = 0·13$, $Q_L = 0·40$; (extract) $Z_L = 0·03$, $Q_L(N) = 1·0$.

Effect of different substrates.

In these experiments whole cells, extracts and cell residues were used; incubation was carried out anaerobically in the presence of 10 m. M substrate (0·5 p.c. in the case of glycogen), 1·0 m. M ATP and 0·5 m. M DPN. The values of Z_L corresponding to the various treatments are set out in Table 1.

TABLE 1.

Lactic acid production (Z_L) from different substrates.

Substrate	Whole cells	Extract	Residue
—	0	·02	·02
glycogen	·07	·03	·05
glucose	·07	·05	·10
fructose	·06	·03	·08
glucose–1–phosphate	·25	·03	·05
glucose–6–phosphate	·38	·05	·10
fructose–6–phosphate	·40	·10	·10
fructose–1:6–diphosphate	·60	·38	·23

Effect of inorganic substances.

In the presence of 10 m. M glucose, 0·5 m. M DPN, 8 m. M nicotinamide and 1·0 m. M ATP the anaerobic production of lactic acid by whole cells was increased by both $MgCl_2$ and KCl. For example, if 5 m. M $MgCl_2$ and 25 m. M KCl were both present the $Z_L = 0·11$; omission of either of the salts depressed the Z_L to 0·09. With extracts, $MgCl_2$ had no effect but KCl raised the $Q_L(N)$ from 1·3 to 1·7.

Action of co-enzymes.

These substances were tested in the presence of a variety of substrates with essentially similar results. DPN decreased the production of lactic acid by whole cells but brought about an increase in the case of extracts. With aerobic experiments on intact protozoa it was possible to observe the motility and shape of the organisms at the end of the incubation period; Table 2 gives results obtained using three different preparations of DPN and in all cases the motility and form of the protozoa were normal after the experiment. Nicotinamide was added in one set in order to stabilize the DPN already present in the cells (Schlenk, 1945). The concentrations of the substances added were glucose, 10 m. M; DPN, 0·5 m. M and nicotinamide, 8 m. M.

TABLE 2.

Action of DPN and nicotinamide on whole cells.

Addition	Z_L
—	0·08
DPN (Sample 1)	0·04
DPN (Sample 2)	0·03
DPN (Sample 3)	0·03
Nicotinamide	0·05

With extracts, however, both DPN and nicotinamide increased the amount of lactic acid formed from either 10 m. M glucose or fructose diphosphate (Table 3).

TABLE 3.

Action of DPN and nicotinamide on extracts.

Addition	Q_L (N) glucose	Q_L (N) fructose diphosphate
—	0·1	22
DPN	0·3	25
Nicotinamide	0·3	25

Table 4 shows the effect of adenyl compounds on anaerobic lactic acid production by whole cells and extracts. Here the incubation mixture contained 0·5 m. M DPN, 8 m. M nicotinamide, 5 m. M $MgCl_2$, 25 m. M KCl and 10 m. M fructose diphosphate. For comparison, results obtained with extracts in the presence of 8 m. M nicotinamide and 10 m. M fructose diphosphate are included.

TABLE 4.

Effect of adenyl compounds.

Compound	Whole cells	Extract
	Z_L	Q_L (N)
—	0·2	15
Adenylic acid (1·0 m. M)	0·3	17
ATP (1·0 m. M)	1·0	17

The concentrations of these coenzymes in the protozoa were estimated enzymatically as previously described. Thus the concentration of DPN was 1·9 mg. p.c. and of adenyl compounds (calculated as adenylic acid) was 24·5 mg. p.c.

Conditions of incubation.

Incubation was carried out both under aerobic conditions in small centrifuge tubes and under N_2 in Thunberg tubes. With extracts, no difference in lactic acid production was observed, but when using whole cells, anaerobic incubation led to greater acid production as compared to aerobic conditions. In N_2 the cells do not survive and are immotile at the end of the 2 hour period; also, the technique of gassing the tubes exposes them for very short periods to low pressures. The increase in glycolysis under anaerobic conditions was always obtained but varied in magnitude. This may be related to differences in resistance to anoxia and temporary low pressures. The inhibition of lactic acid production caused by air, ranged from 15 to 65 p.c.

Action of inhibitors.

Iodoacetic acid, sodium azide and potassium cyanide were tried as possible inhibitors of lactic acid production. In Table 5 are set out the results of an experiment on anaerobic acid production by whole cells; the reaction mixture contained 0·5 m. M DPN, 8 m. M nicotinamide, 1·0 m. M ATP, 10 m. M glucose, 5 m. M $MgCl_2$ and 25 m. M KCl.

TABLE 5.

Effect of inhibitors.

	Z_L
Control	0·23
1·0 m. M iodoacetate	0·02
0·1 m. M iodoacetate	0·05
0·1 m. M azide	0·22
10 m. M cyanide	0·21

Thus only iodoacetate produced marked inhibition; azide could not be used in higher concentrations because it interfered with the determination of lactic acid.

DISCUSSION.

Course of glycolysis.

Many different acidic products are formed during the course of the metabolism of carbohydrate by protozoa. Most of the investigations have been carried out with parasitic organisms; with regard to ciliates, von Brand (1946) gives some details of unpublished work by Thomas which shows that *Tetrahymena geleii* can convert glucose into either lactic acid or a mixture of acetic and succinic acids. Thomas suggests that the two groups of acidic products are formed by different and independent processes. Cunningham and Kirk (1941) showed that another ciliate, *P. caudatum*, can produce acid aerobically from starch or glucose; the acid was not identified but reported as H^+. Recalculation of their data gives a value for the Q_L of 0·5, which is similar to the values obtained in the present work. In Table 6 figures for glycolytic rates in other invertebrates (Humphrey 1949, 1950; O'Brien, unpublished) are compared with those for *P. caudatum*.

TABLE 6.

Comparison of glycolytic rates.

	Tissue	$Q_{pyruvate}$	$Q_{lactate}$
P. caudatum	whole cells	0	0·40
Allolobophora sp.	body muscle	0·70	5·0
Periplaneta americana	leg muscle	0·42	0·25
Locusta migratoria	wing muscle	1·5	0·5
Saxostrea commercialis	nacreous adductor muscle	0·002	0·06
Saxostrea commercialis	vitreous adductor muscle	0·04	0·16

The Z_L for *P. caudatum* extracts was much lower than for whole cells; the glycolytic rate with glucose as the substrate could not be increased by traces of fructose diphosphate (0·1–0·2 m. M) above that of the glucose or fructose diphosphate alone.

Activators.

In the intact cell the spatial distribution of salts and coenzymes is probably such as to give maximal possible efficiency of metabolic processes. To show the full dependence of a given mechanism upon an essential component, the system should preferably be depleted partially or fully of that component. In this series of experiments no attempt was made to reduce the concentration of any substance in the cells or extracts. Considering together the results on cells and extracts it seems that added K, Mg, DPN and ATP are needed for maximal rates of glycolysis. The concentration of DPN has been determined in many species (Ochoa and Ochoa, 1937; Hutchens *et al.*, 1941; Barron and Tahmisian, 1948 and Seaman, 1949) and these figures are compared with each other in Table 7. The results obtained by spectrophotometric means alone, are almost certainly too high, owing to the lack of specificity of this method.

TABLE 7.

DPN concentration in different species.

Species	mg. p.c. DPN	Method
P. caudatum	1·9	enzymic
Colpidium campylum	8·2	enzymic
Chilomonas paramecium	8·5	enzymic
Coelenterates	3	photometric
Echinoderms	8	photometric
Gephyrean worms	17	photometric
Molluscs	30	photometric
Marine Arthropods	45	photometric
Periplaneta americana	27	enzymic
Rat	20	enzymic

Thus protozoa in general and *P. caudatum* in particular, have a low concentration of DPN.

Inhibitors.

The lower acid production under aerobic as compared to anaerobic conditions indicates the operation of a Pasteur effect. Stockdale (unpublished) has found that whole cells of the same organism show an increased O_2 consumption in the presence of pyruvic and lactic acids and this supports the idea that under aerobic conditions there is less acid accumulation since then, part of the acid product is oxidatively metabolised.

The experiments with iodoacetic acid demonstrate that glycolysis is inhibited by this agent. Thus of the invertebrates studied, *P. caudatum*, *S. commercialis* and *Allolobophora sp.*, (O'Brien, unpublished) show decreased glycolysis in the presence of iodoacetate whereas two species of insects (*L. migratoria* and *Peri-*

planeta americana) possess glycolytic mechanisms which are unaffected by this compound (Humphrey, 1949).

Cyanide and azide inhibit the O_2 consumption of *P. caudatum*, by about 50 p.c. (Humphrey and Humphrey, 1948) but they seem to have little inhibitory effect on glycolysis.

SUMMARY.

P. caudatum glycolyses glycogen, glucose, fructose, glucose-1-phosphate, glucose-6-phosphate and fructose-1:6-diphosphate with the production of lactic but not pyruvic, acid. The intensity of the anaerobic lactic acid production from glucose is about 0·4 μl./mg. dry weight/hour or 0·13 μl./10^4 cells/hour.

Mg, K, DPN, ATP, adenylic acid and nicotinamide can each increase lactic acid production under suitable conditions.

The concentration of DPN in *P. caudatum* is 1·9 mg. p.c. and of adenyl compounds is 24·5 mg. p.c.

Iodoacetic acid and aerobic conditions each lower the glycolytic rate; KCN and NaN_3 produce no significant effect.

These results are compared with similar findings on other animals.

REFERENCES.

Barron, E. S. G. and Tahmisian, T. H. (1948): J. cell. comp. Physiol., 32, p. 57.
von Brand, T. (1946): Anaerobiosis in Invertebrates. Biodynamica, Missouri.
Conway, E. J. and Cooke, R. (1939): Biochem. J., 33, p. 479.
Cunningham, B. and Kirk, P. (1941): J. cell. comp. Physiol., 18, p. 299.
Harvey, S. C. (1949): J. biol. Chem., 179, p. 435.
Humphrey, G. F. (1949): J. cell. comp. Physiol., 34, p. 323.
Humphrey, G. F. (1950): Austral. J. exp. Biol., 28, p. 151.
Humphrey, B. A. and Humphrey, G. F. (1948): J. exp. Biol., 25, p. 123.
Hutchens, J. O., Jandorf, B. J. and Hastings, A. B. (1941): J. biol. Chem., 138, p. 321.
Kerr, S. E. (1941): *Ibid.*, 139, p. 131.
Le Page, G. A. (1947): *Ibid.*, 168, p. 623.
Ochoa, S. and Ochoa, C. G. (1937): Nature, 140, p. 1,097.
Racker, E. (1950): J. biol. Chem., 184, p. 313.
Schlenk, F. (1945): Adv. Enzymol., 5, p. 207.
Seaman, G. R. (1949): J. cell. comp. Physiol., 33, p. 1.

THE GROWTH OF LEPTOSPIRAE IN SEMI-SOLID MEDIA

by J. J. LAWRENCE

(From the School of Public Health and Tropical Medicine, University of Sydney).

(Accepted for publication 20th February, 1951.)

Leptospirae, when grown in a semi-solid medium, multiply only in the surface layers. The opacity produced by their growth extends from the surface downwards and ends, usually sharply, at a depth of about one centimetre. A thin opaque white disc may often be seen where this macroscopic growth ends and is caused by the great numbers of leptospirae, which are present in a concentration far greater than in the upper layers of the medium. Therefore this narrow zone seems to provide the optimum conditions for their multiplication.

Noguchi (1928) noted that leptospirae tended to multiply more abundantly in a semi-solid particle, such as a piece of agar or fibrin, than in a clear liquid medium; and that the addition of 0·1-0·3 p.c. agar furnished the optimum semi-solid condition for abundant growth. He continued: "Too much agar must not be used, for the organisms are obligatory aerobes, and the depth and rapidity of penetration of oxygen into the medium are less the more agar there is added. With a concentration of 0·3 p.c. or less, the surface layer of 2-3 cm. is easily penetrated by oxygen, as can be demonstrated by using methylene blue as an indicator. The leptospira itself may be used as an indicator of the penetration of oxygen into the medium for it grows only in the aerated zone, the same zone to which the colour of methylene blue is found to return after removal of a vaseline seal." However, Rahn and Richardson (1941), by observing the rate at which leuco methylene blue is reoxidised, have shown that oxygen diffuses fairly rapidly into a nutrient agar medium even when the concentration of agar is high; in fact it diffuses through 0·1 p.c. agar only about twice as fast as through 5 p.c. agar. Also leptospirae multiply slowly; for example Chang (1947) has shown that their generation time is about two days. It seems possible that sufficient oxygen to enable the leptospirae to grow would be supplied by diffusion even if the medium contained enough agar to make it quite stiff, say the 2 p.c. commonly used in bacteriological media. Perhaps higher concentrations of agar offer a barrier to the leptospirae rather than to the oxygen. In any case, if the character of the growth of leptospirae in semi-solid media is to be explained solely in terms of their oxygen requirements it seems necessary to assume that the optimum oxygen tension is less than that of air in order to explain the dense ring of growth some distance below the surface—the organisms are microaerophilic rather than strictly aerobic. Dinger (1932) became inter-

ested in this phenomenon of a ring of growth because he believed that it was connected with the maintenance of virulence of leptospiral cultures. (He had previously found that cultures maintained their virulence much better in semi-solid than in liquid media.) At first he thought that the existence of this ring showed that leptospirae were microaerophilic but further experiments convinced him that the ring was at the level of the minimum oxygen tension that would support growth and that this minimum was not the optimum. His explanation of the increased growth at this level of minimum oxygen tension was that the metabolic products could diffuse away into the depths of the medium where no growth was taking place.

However, a need for an increased carbon dioxide tension might explain this ring of growth as it does the "band phenomenon" shown by *Br. abortus* (Wilson, 1931). Growth may be less intense at the surface because there carbon dioxide is being given off into the atmosphere, while in the depths of the medium no growth may take place because there the conditions are anaerobic. It seemed worthwhile to re-investigate the oxygen requirements of leptospirae.

Materials and Methods.

Several species of leptospirae were used but most of the work was done with a strain of *Leptospira pomona* Derrick. The other species used were *L. icterohaemorrhagiae* (Inada and Ido) (two strains from the National Collection of Type Cultures: Nos. 6,805 and 6,806: and the Jackson strain), *L. mitis* Johnson and *L. canicola* Schüffner.

The culture medium used was either the semi-solid agar medium due to Fletcher (1928), which consists of 1 part of 2 p.c. nutrient agar diluted with about 10 parts of water, rabbit serum being added to give a final concentration of 15 p.c., or Vervoort's medium (Van Thiel, 1948); a buffered 0·15 p.c. peptone water plus about 10 p.c. rabbit serum. The medium was dispensed in 5 ml. amounts usually in 5 × ½ inch test-tubes and then heated at 56° C. for half an hour. The cultures were incubated at 28–30° C.

For those experiments that involved incubation in an atmosphere other than air the cultures were placed in a McIntosh and Fildes jar which was then partially evacuated. Oxygen, hydrogen or carbon dioxide was then run in to restore the pressure to normal and provide the required tension of oxygen or carbon dioxide. At the end of the experiment a sample of gas was withdrawn and analysed.

To count the leptospirae, a Petroff-Hausser counting chamber was used. The culture was usually diluted 1/20 with buffered formolized saline for the count.

RESULTS AND DISCUSSION.

The first problem attacked was the rate of diffusion of oxygen into the semi-solid medium. Methylene blue was added to Fletcher's medium at a concentration of 1/200,000 or 1/500,000 and then reduced by keeping the tubes of culture medium under anaerobic conditions in a McIntosh and Fildes jar. When the methylene blue had been completely decolorized the tubes were removed from the jar and kept at 30° C. under atmospheric conditions. The surface layer became reoxidised at once and the depth of this blue zone of oxidised methylene blue steadily increased: after 2½ hours it was 12 mm.; after 5 hours, 17 mm.; after 8 hours, 21 mm.; after 10 hours, 23 mm.; and after 24 hours, 35 mm. In agreement with the law of diffusion the depth of the blue zone when plotted against the square root of the time gives a straight line.

It follows that the inability of leptospirae to grow in the depths of the medium is not due to failure of oxygen to diffuse there. However, the oxygen may be used up by the leptospirae in the surface zone of growth quickly enough to stop growth in the lower part of the medium. This can be tested by adding a suitable oxidation-reduction indicator to the medium and seeing whether it is decolorized in the depths of the medium when growth takes place. Methylene blue is unsuitable as it is toxic for leptospirae.

As a preliminary the oxidation-reduction potential of the zone of growth in the upper part of the tube and also in the depths of the medium was determined by the use of platinum electrodes and a vacuum tube electrometer. The electrodes were left in position and readings were taken each day. In a series of four experiments with *L. pomona* and *L. icterohaemorrhagiae* the Eh in the zone of growth varied from 0·2 to 0·35 volts while the Eh in the depths of the medium reached a minimum of —0·05 to —0·075 volts. Attempts to measure the Eh at a point just below the Fletcher's ring were not a success. The pH of the cultures was measured by means of a glass electrode at the end of the experiment (after some 14 or more days' incubation) and varied from 7·6 to 7·8. Control experiments in which indicators were incorporated in the medium did not show any appreciable change in pH during the growth of leptospirae. This has also been the experience of other workers even when glucose was added to the medium (Chang, 1947).

On the basis of these results a number of oxidation-reduction indicators whose E_0' seemed suitable were tested by incorporating them in the Fletcher's medium at a concentration of 1/20,000 and 1/50,000. The medium was then inoculated with leptospirae and incubated at 30° C. for at least two weeks. The indicators and their E_0' at the pH of the medium in this experiment (pH 7·7) were indigo disulphonate, —0·155 v.; indigo trisulphonate, —0·11 v.; 1-naphthol sodium sulphonate indo 2, 6-dichlorophenol indophenol, 0·07 v.; and 2, 6-dichlorophenol indophenol, 0·168 v. With indigo disulphonate there was no evidence of reduction in any part of the tube. As the Eh of this indicator at pH 7·7 is —0·095 v. when 99 p.c. oxidised, the Eh in all parts of the tube should be greater than this. The indigo trisulphonate was about half reduced in the lower part of the tubes indicating an Eh of —0·11 v. It will be remembered that the Eh found by the platinum electrode in the depths of the medium was —0·05 to— 0·075 v. which compares quite well with the results found with these two indicators. The 1-naphthol sodium sulphonate indo 2, 6-dichlorophenol is reduced at the bottom of the tube but this reduction reaches the level of the leptospiral growth only if the strain is very quick growing—more usually the blue colour of the oxidised indicator extends for a few millimetres below the lower limit of growth. On the other hand the 2, 6-dichlorophenol indophenol is reduced just at this level but if the strain is not growing well enough to form the ring, even though the haze of growth ends sharply, the blue colour of oxidised indicator will extend for some millimetres below the level of macroscopic growth. The Eh of this indicator at pH 7·7 is 0·01 v. when 99 p.c. reduced and 0·13 v. when 99 p.c. oxidised.

This last indicator, used at a concentration of 1/50,000, was found to be very suitable for work with leptospirae. In cultures inoculated in the usual way by adding a few drops of a well grown culture to the surface, reduction begins 0·5 to 1 cm. below the surface (the final limit of growth is in this region) and spreads downwards to the bottom of the tube as growth proceeds. If the tube is inoculated by the shake culture technique reduction of the indicator proceeds from the bottom of the tube, as would be expected. In cultures of some strains the indicator becomes re-oxidised below the level of growth after some weeks; the blue

colour slowly creeps down until the bottom is reached; presumably the leptospirae are no longer metabolising actively although they are still present and alive in the surface layer as may be seen by darkground examination.

Two of the cultures of *L. icterohaemorrhagiae* grew particularly quickly and showed, besides the usual haze of growth at the surface, a fine ring of leptospirae which, starting just below the lower limit of the haze of growth, moved down the tube and finally disappeared. When the dichlorophenol indo-phenol was added to such cultures it was seen that this fine ring of growth was near the top of the bottom zone showing oxidised indicator and, as reduction proceeded in this zone, the ring moved down the tube so as to remain in a region where oxygen was still present. Those strains of leptospirae that did not show this phenomenon presumably could not grow quickly enough to show visible growth before the oxygen in this zone was used up. It may be mentioned here that, with all the strains tested, leptospirae could always be found below the surface zone of growth, even right at the bottom of the tube, but their numbers were too few for growth to be macroscopically apparent.

All these results reinforce the belief that leptospiral growth is limited to the surface zone because of a need for oxygen. However they do not explain the marked increase in concentration of leptospirae which leads to the formation of a definite ring at the very bottom of this surface zone of growth.

To see whether this phenomenon could be explained in terms of an optimum oxygen tension for the leptospirae, cultures were incubated in jars that contained various amounts of oxygen. Some of the cultures contained 2, 6-dichlorophenol indophenol which acted as an early indicator of growth, or at least of metabolism. None of the strains would grow under anaerobic conditions or in the presence of 100 p.c. oxygen. Two of the strains of *L. icterohaemorrhagiae* (those received from the National Collection of Type Cultures) would grow when the concentration of oxygen was as low as 0·5 p.c. or as high as 90 p.c. The other strains seemed more fastidious but would grow when the oxygen was varied from 2·7 p.c. to 50 p.c. at least. When the concentration of oxygen was low, growth was confined to a zone near the surface, the ring forming a few millimetres below the surface, but when it was as high as 50 p.c. the zone of growth extended down about three times as far as usual. The cultures containing 2, 6-dichlorophenol indo-phenol showed that oxygen was limited to the zone of growth, as the indicator was sharply reduced at the lower limit of the zone of growth.

While these results underlined the importance of oxygen for the growth of leptospirae they did not indicate that the leptospirae grew better at an oxygen tension below that of air. Counts showed that the concentration of leptospirae in a fluid medium (Vervoort's) incubated at a lowered oxygen tension was no higher than in control cultures incubated in air. Nor did they support the idea that the ring was caused by a need for increased CO_2 tension for maximum growth.

An attempt was made to test this directly by incubating cultures in an atmosphere containing 3·5 p.c. CO_2 using a buffered Fletcher's medium. Three of the strains grew well and showed the ring in the usual position. There was no tendency for it to be nearer the surface or thicker as would be expected if increased CO_2 were a necessity for its formation. The pH of the cultures immediately on removing them from the jar was 6·6–6·8 which probably explains the failure of some strains to grow.

Finally, the explanation of the ring formation put forward by Dinger remains, namely that the ring is formed at a zone where sufficient oxygen for

multiplication is present and where the products of metabolism can diffuse away into the medium. Dinger claimed that leptospirae would show this ring in a liquid medium which lay above a column of 3 p.c. agar, as then the metabolic products could diffuse away. The ring, of course, lay at the junction of the agar and the liquid medium.

An attempt was made to confirm this by putting 5 or 10 ml. of 3 p.c. agar in Vervoort's medium in tubes and overlaying it with ordinary Vervoort's medium to a depth of 0·6 to 2 cm. However, it was impossible to be sure that there was a ring at the interface of the agar and the liquid medium caused by increased growth of the leptospirae, as the meniscus of the agar appeared as a whitish ring. But if growth is improved by enabling the metabolic products to diffuse away, the concentration of leptospirae in the fluid overlay should be greater than that in control tubes filled to the same depth with Vervoort's medium. This was found to be the case. The concentration of leptospirae in the overlay was from two to ten times as great as in the control tubes. This result was confirmed by growing the leptospirae in cellophane bags containing 5 ml. of Vervoort's medium and surrounded outside by 50 to 100 ml. of the same medium. Counts of these leptospirae showed that their concentration in the bags was about twice that in the control tubes.

We may conclude that leptospirae are strict aerobes, which explains the limitation of their growth to the surface zone in semi-solid cultures, and that the increased concentration of leptospirae at the bottom of the zone of growth, which is often apparent as a definite ring, is due to the fact that in this region metabolic products can diffuse away into the depths of the medium.

SUMMARY.

Leptospirae are strict aerobes but will grow in atmospheres with a wide range of oxygen tension. At least some strains will grow in atmospheres whose oxygen content varies from 0·5 p.c. to 90 p.c.

Leptospirae reduce 2, 6-dichlorophenol indophenol, which is a sensitive indicator of their growth.

The ring of enhanced growth, which is seen in cultures of leptospirae in a semi-solid medium at the bottom of the zone of growth, and which sharply demarks the limit of macroscopic growth, is due to two factors. The first is that leptospirae are strict aerobes and, as all the oxygen is used up in the surface zone no growth can take place below this ring; and the second is that their metabolic products can diffuse away into the unoccupied depths of the tube. It follows that this narrow zone is particularly suited to the growth of leptospirae.

REFERENCES.

Chang, S. L. (1947): J. infect. Dis., 81, p. 35.
Dinger, J. E. (1932): Gen. Tschr. Ned. Ind., 72, p. 1,511.
Fletcher, W. (1928): Trans. Roy. Soc. trop. Med., 21, p. 265.
Noguchi, H. (1928): The Newer Knowledge of Bacteriology and Immunology, Ed. Jordan, E.O., and Falk, I. S., chapter 36, p. 473, Chicago, University of Chicago Press.
Rahn, O. and Richardson, G. L. (1941): J. Bact., 41, p. 225.
Van Thiel, P. H. (1948): The Leptospiroses, Leiden Universitaire Pers Leiden.
Wilson, G. S. (1931): Brit. J. exp. Path., 12, p. 152.

SOME INVESTIGATIONS ON LYMPH FROM A THORACIC DUCT FISTULA IN MAN

by F. C. COURTICE, W. J. SIMMONDS AND A. W. STEINBECK

(From the Kanematsu Memorial Institute of Pathology, Sydney Hospital, Sydney).

(Accepted for publication 7th March, 1951.)

Progressive loss of weight and hypoproteinaemia occur in man when the thoracic duct lymph does not return to the circulation. The lymph may be lost from a fistula of the thoracic duct in the neck (Crandall, Barker and Graham, 1943), or may escape into a serous cavity as in chylothorax (MacNab and Scarlett, 1932). The rates at which fluid and protein may be lost in thoracic duct lymph have been shown to be rapid in acute experiments in animals; for example, Courtice (unpublished) found in nembutalized dogs that the rate of escape of fluid and protein in the lymph was equivalent to a daily loss of 60 p.c. of the plasma volume and 45 p.c. of the circulating plasma proteins. In fistulae of the human thoracic duct, few measurements have been made of the loss of fluid and protein in the lymph.

An opportunity to obtain further data in man arose recently in a subject with a post-operative fistula of the thoracic duct in the neck. In addition to the output of fluid, protein and lymphocytes, which are of interest for comparison with previous results in man and animals, it was decided to measure the amount of T1824 (Evans' Blue) recovered in the lymph for the first hour after intravenous injection. Return of T1824 to the circulation by the lymph during the estimation of blood volume would introduce an error in the calculations. Cardozo (1940–41) and Courtice (1943) found that negligible amounts of T1824 were recovered in thoracic duct lymph in anaesthetized animals in the first hour after intravenous injection of dye but the experiments of Ferrebee, Leigh and Berliner (1941) are considered by some to contradict these findings. The point has not previously been investigated in man.

METHODS.

The simplest and most reliable method of collecting the lymph was found to be continuous aspiration from the fistula by sterile Pasteur pipettes. All clots were cleared away and all lymph removed from wound and fistula before beginning a period of collection. Since damming back of lymph by clots in the fistula would result in a temporary increase of flow on commencing drainage, quantitative collection was not begun until lymph had been flowing freely for some time. Lymph was transferred immediately after aspiration into vessels containing dry

heparin to prevent clotting. A fresh vessel was used every ten minutes, without interrupting the collection of lymph. At the end of the experiment the volume collected in the successive ten minute periods was measured. A portion of each lymph sample was then taken for cell counts, protein and dye estimation.

Differential white cell counts were made on venous blood films by counting at least 500 cells. Differential counts on lymph films were unsatisfactory. However, examination of diluted lymph showed that virtually all the cells present were mononuclear leucocytes, presumably lymphocytes.

The protein and non-protein nitrogen concentrations of lymph and plasma were determined by microKjeldahl digestion and Nesslerisation. The colour density was estimated with the Spekker photoelectric absorptiometer using an Ilford 603 and Calorex heat filter. For the non-protein nitrogen estimation the proteins were precipitated with trichloracetic acid. The albumin fraction was estimated after precipitation of the globulins and fibrinogen with 21 p.c. sodium sulphite (Campbell and Hanna, 1937). The method of estimating dye concentration in lymph and plasma has been described previously (Courtice and Steinbeck, 1950). The method employed, extraction of dye into n-butyl alcohol (Harington, Pochin and Squire, 1940), eliminates the variation in opacity which occurs from sample to sample, especially in lymph.

RESULTS.

Mrs. F., aged 34 years, was admitted to Sydney Hospital on 2nd December, 1950, for resection of a malignant melanoma on the left side of the neck and block dissection of the lymph nodes. During operation on 5th December, the main thoracic duct or a large branch, pursuing an anomalous course, was severed. Ligation apparently stopped the leak and immediate post-operative recovery was uneventful. From the third post-operative day onwards, however, a copious creamy discharge which clotted readily was noticed at the site of the drainage tube. It was decided to close the fistula on 16th December, and the patient agreed to a collection of the chyle being made for experimental purposes on 15th December.

On 16th December, 1950, the wound was packed with "oxycel" gauze together with thrombin "topical" application. The treatment was not immediately effective, free drainage of the lymph continued and the patient lost weight. By 2nd January, 1951, after further repacking, chylous leakage had virtually stopped. The patient's general condition improved and skin grafting was possible on 9th January.

TABLE 1.

The plasma protein concentration, weight and white cell count of Mrs. F.

Date	Remarks	Plasma proteins gm. p.c.	Weight	White cell count	
			Stone lb.	Total /c.mm.	Lymphocytes /c.mm.
3/12/50		—	8 . 5	—	—
5/12/50	Operation	—	—	—	—
15/12/50	Lymph collected	6·0	—	14,200	940
16/12/50	Wound packed	—	—	—	—
2/1/51	Leakage stopped	—	—	—	—
7/1/51		—	7 . 1	12,000	1,080
13/1/51		—	7 . 12	—	—
16/1/51		6·8	—	5,200	1,090
18/1/51		—	—	5,500	935
26/1/51		7·1	—	5,200	1,040
7/2/51		7·4	8 . 0	5,650	1,322

When last seen on 7th February, 1951, the general improvement had been maintained.

The clinical progress has been summarized in Table 1. It can be seen that the plasma proteins were low and that the patient lost weight while the fistula was draining freely. During this period a diet rich in protein, fat and vitamins was given and a total of 2 litres of serum together with 0·5 litres of blood infused on different occasions. After the chylous leakage stopped there was a rapid gain of weight and a rise in plasma protein concentration. The lymphocyte count in the blood remained at a low normal value.

The subject was available for physiological study only on the day before operation for closure of the fistula. It was decided, therefore, to collect lymph during two periods that day, one in the mid afternoon and one in the evening, and for a short period on the following morning when the patient would be fully post-absorptive and anaesthetised.

Lymph was first collected for a period of 85 minutes on the afternoon of 15th December, 1950. Collection began 2¼ hours after lunch which consisted of fish, vegetables, junket, custard and pastry. Twenty-five minutes after beginning the experiment, 28 mg. of T1824 in 0·5 p.c. aqueous solution were injected intravenously. Every ten minutes for a further 60 minutes the volume of lymph was measured and a sample taken for measurement of dye concentration. Venous blood samples were taken immediately before, and 1 and 5½ hours after injection of the dye. Table 2 shows the results obtained in this experiment. The total volume of lymph collected in 85 minutes was 75·9 ml., which is equivalent to a flow of 55 ml./hr. or 1·1 ml./kg./hr. The protein concentration of the lymph was steady throughout with an average of 3·53 gm. p.c., which gives an hourly output of protein in the lymph of 1·89 gm. or 1·4 p.c. of the circulating plasma-proteins. The lymphocyte count averaged 2,930 per c.mm., equivalent to an hourly output of lymphocytes of 157 millions or 4·3 p.c. of the circulating lymphocytes. After the intravenous injection of dye, no dye appeared in the lymph in the first 20 minutes and during the first hour after injection the lymph contained only 0·04 mg. of dye or 0·14 p.c. of the amount injected.

TABLE 2.

The flow of lymph, output of protein and of lymphocytes from fistula of thoracic duct in the neck together with rate of recovery of the blue dye, T1824, in lymph after intravenous injection. Experiment began 2¼ hours after lunch on 15/12/50.

	Lymph								Blood and plasma at end of experiment
	Before injection of dye		After injection of dye						
	Minutes		Minutes						
	0–15	15–25	0–10	10–20	20–30	30–40	40–50	50–60	
Volume of lymph ml.	11·8	4·5	5·5	9·8	9·6	12·5	11·8	10·4	—
Protein concentration gm. p.c.	3·35	3·71	3·60	3·48	3·54	3·60	3·60	3·35	6·07
Albumin concentration gm. p.c.	2·26	—	2·34	—	2·45	—	—	2·45	3·53
Dye concentration mg./litre	0·0	0·0	0·0	0·0	0·4	0·5	1·2	1·5	12·1
Lymphocyte count thousands/c.mm.	3·2	3·8	2·18	2·05	3·1	3·55	3·07	2·48	0·75

Lymph was collected during a second period on 15th December, 1950, two hours after the evening meal which consisted of oysters, lettuce, egg flip, stewed chops, bread, butter and tea. The results of this experiment are summarized in Table 3. The lymph flow was more rapid during this period of collection, being on an average 78 ml./hr. or 1·6 ml./kg./hr., and it was more milky in appearance. The average protein content of the lymph was, however, lower at 2·99 gm. p.c. with an hourly output of 2·33 gm. or 1·8 p.c. of the circulating plasma protein. The lymphocyte count was also lower with an average of 2,000 per c.mm., but the average hourly output of lymphocytes was 156 millions, the same as in the earlier period.

TABLE 3.

The flow of lymph and output of protein and lymphocytes from fistula of thoracic duct in neck two hours after the evening meal on 15/12/50.

	Lymph				Blood and plasma at end of lymph collection
	Minutes				
	0–10	10–20	20–30	30–40	
Volume of lymph ml.	15·80	14·0	13·60	8·40	—
Protein concentration gm. p.c.	2·80	3·15	2·99	2·99	5·94
Albumin concentration gm. p.c.	1·64	—	—	1·89	3·46
Lymphocyte count. Thousands/c.mm.	2·18	1·88	2·10	1·85	1·02

On the morning of 16th December, 1950, lymph was collected for a period of 5 minutes only, in the operating theatre when the patient was post-absorptive and anaesthetized with pentothal. The volume of lymph collected was 4·4 ml., equivalent to a flow of 53 ml./hr.; the protein concentration was 3·71 gm. p.c. and the white cell count 2,710/c.mm. Although the patient had had no food for 13 hours, the lymph appeared milky but less so than in the previous samples.

DISCUSSION.

Before considering the data obtained from these experiments it is necessary to state the grounds for believing that all or most of the thoracic duct lymph was escaping by way of the fistula. At operation either the main duct or a large branch was cut—lymph spurted to a height of ten inches and the flow was considerable until the vessel was ligatured. The persistent escape of large volumes of chyle after the operation despite repeated firm packing of the fistula suggests that lymph was escaping from a large vessel, and that there were no intact branches or collaterals of sufficient size available to carry away the lymph. Glenn, Cresson, Bauer, Goldstein, Hoffman and Healey (1949) showed that the plasma lipids did not rise after a fatty meal in unanaesthetized dogs with a complete fistula of the thoracic duct and the right lymph duct tied, whereas after the fistula was closed there was a rise in plasma fats comparable to that in the same animals with all lymph ducts intact. It was not possible to arrange a comparable experiment in the case we have studied but it was noted that the opalescence of the plasma appeared the same in the evening,

when the lymph was very milky and a thick layer of cream formed on standing, as it was in the mid-afternoon when the lymph was much less milky. The volume of lymph collected and the relatively steady rate of flow suggest that the greater part of the lymph was being collected without blockage in the fistula during the period of experiment. The subnormal plasma protein concentration and the loss of weight while lymph was draining from the fistula followed by a rise in plasma protein concentration and gain in weight when drainage ceased are also suggestive of loss of the majority of the thoracic duct lymph.

Volume flow.

To assess the total volume of lymph which may pass through the human thoracic duct in the course of a day it is necessary to know the rates of flow

TABLE 4.

Lymph flow and protein and lymphocyte output from the thoracic duct in man compared with results obtained with animals, at rest but under varying dietary conditions.

		Rate of flow ml./kg./hr.	Protein		Lymphocytes		
			gm. p.c.	gm./kg./hr.	10^3/c.mm.	10^6/kg./hr.	Daily output Total in blood
Mrs. F		1·1–1·6	2·8–3·7	0·036–0·044	1·9–3·8	3·1	1·0
J. W.	Crandall *et al.*	1·4	3·0–5·0	0·047–0·060	—	—	—
Dog	Nembutal anaesthesia Courtice	1·2	4·1	0·051	6·8 (2·7–12·2)	6·7	1·5
Dog	Local anaesthesia Petersen and Hughes	2·2	4·7	0·102	—	—	—
Dog	Chloralose anaesthesia Yoffey	—	—	—	—	21·2	2·1
Cat	Nembutal anaesthesia Simmonds	2·1	4·7	0·100	—	—	—
Cat	Dial and urethane anaesthesia Adams, Saunders and Lawrence	2·6	—	—	14·2 (4·3–29·6)	35·5	2·4
Cat	Decerebrate Sanders, Florey and Barnes	1·0	—	—	13·5 (5·9–28·3)	14·6	1·0 (0·5–3·5)
Rabbit	Nembutal anaesthesia Sanders, Florey and Barnes	1·9	—	—	35·0	66·7	5·0
Rat	Unanaesthetized Reinhardt	2·0	—	—	19·0 (6·7–46·3)	38·0	2·7

For further data see Drinker and Yoffey (1941).

of thoracic duct lymph under such conditions as digestion, sleeping, exercise and sedentary activity. It is difficult to close chylous fistulae permanently by firm packing, but the chyle clots readily, causing temporary obstruction to flow even in unpacked fistulae. It has, therefore, never been practicable to obtain lymph continuously over very long periods from the human being. The nearest approach to the problem is provided by considering the samples collected for relatively short periods under various conditions in man and animals.

In Table 4 the results in the case of Mrs. F. are summarized together with data calculated from experiments recorded in the literature. The rate of flow of thoracic duct lymph, per kg. body weight, in man at rest some hours after a meal, assessed from the cases of Mrs. F. and Miss J. W. (Crandall *et al.*, 1943), is comparable to that in animals and equivalent to a daily volume of 50 to 80 p.c. of the fluid in the circulation. In Crandall *et al.*'s patient (1943) the lowest rates of flow were about 30 p.c. of this value and occurred at the end of some hours' practical immobility. The highest rate of flow occurred for an hour and a half after a heavy meal when lymph flow was 1½ to 3 times the value in Table 4. They considered that the rate of flow varied mainly with the rate of intestinal absorption and that activity of the skeletal muscles or changes in gastrointestinal motility had little effect. The effect of exercise on the flow of thoracic duct lymph in man is not known. Cain, Grindlay, Bollman, Flock and Mann (1947) found a threefold increase with short periods of exercise in unanaesthetized dogs but it is difficult in short experiments to assess the part played by increased expression of lymph from the lymphatics. In the case of Mrs. F. a few deep breaths, talking, sitting up, changing her position in bed and swallowing fluid resulted in an increased rate of flow from the fistula. It has been repeatedly shown that deep breathing increases the rate of flow of thoracic duct lymph in animals, even if continued for an hour or more. Since the volume of the lymph pool which empties into the thoracic duct is unknown, it is difficult to say whether such increases of thoracic duct lymph flow are due to an increase of lymph formation or to an increased expression of lymph from the vessels. In the latter case the increase might be balanced over the course of a day by a later decrease in flow as the lymph pool refilled.

From what is known, then, of the rate of flow of thoracic duct lymph the average flow throughout the day would probably be between 1 and 2 ml./kg./hr., that is a daily flow of 24–48 ml./kg. In both man and animals, since the plasma volume is about 40–50 ml./kg., it is clear that the volume of fluid lost daily in thoracic duct lymph fistulae could hardly be less than 50 p.c. of the plasma volume and might well be nearer to 100 p.c. of the circulating plasma.

Protein output.

The loss of protein from thoracic duct fistulae is an even greater threat to the bodily economy than the loss of fluid. Crandall *et al.* (1943) made the first reliable determinations of protein concentrations in human thoracic duct lymph. With the exception of a sample taken after their patient had vomited, the protein concentrations range from 3 to 5 gm./100 ml. In the case of Mrs. F. the protein concentrations were from 2·8 to 3·7 gm./100 ml. in different lymph samples. Her plasma volume was 2·17 litres and the rate of protein output during the 3 periods of experiment was equivalent to a daily loss of 34 to 40 p.c. of the circulating plasma proteins. The magnitude of the

extravascular circulation of protein is worthy of emphasis. If the protein output of the other major lymph channels in man is comparable to that in animals, they should account for a daily return to the blood stream of a further 8 p.c. or more of protein which has leaked out of the capillaries. It is possible, of course, that not all the protein returning in the thoracic duct lymph has passed out of the capillaries. Drinker (1945) has suggested that plasma protein newly formed in the liver may reach the blood stream by way of the thoracic duct. If this is so, it is surprising that plasma protein concentration in cases of thoracic duct fistulae does not fall even more rapidly.

Output of lymphocytes.

In the case of Mrs. F. the leucocytes numbered 1,850 to 3,800/c.mm. in thoracic duct lymph. Differential counts on lymph were unsatisfactory but it was obvious in the counting chamber that practically all the leucocytes were mononuclear. In normal thoracic duct lymph in animals virtually all the mononuclear cells are lymphocytes. Mrs. F.'s blood volume was 3·88 litres and the total lymphocyte count in her blood ranged from 750 to 1,090 per c.mm. Lymphocytes were therefore being lost in thoracic duct lymph at a rate of 85 to 130 p.c. of the total circulating lymphocytes per day. There appear to be no other determinations of lymphocyte output in man save that of Heidecke (1906) who found the lymphocytes numbered from 2,000 to 25,000/c.mm. in chylous fistula lymph. The lymphocyte count and the total output of lymphocytes in animals varies enormously not only in different animals of the same or different species, but also in the same individual animal during the course of an experiment or at different times after a meal. In spite of these wide variations, however, it is evident from Table 4 that the ratio of output of lymphocytes to the total circulating lymphocytes is of the same order in man and animals; that is, on an average the lymphocyte stays in the blood for a period of 5 to 24 hours.

In animals, ligation of the major lymph channels results in a marked fall in the circulating lymphocytes (Lee, 1922), and in complete lymphatic obstruction the lymphocytes practically disappear (Blalock, Robinson, Cunningham and Gray, 1937). It is difficult to understand why the lymphocytes in the blood in Mrs. F. remained unchanged at a low normal level after the fistula had ceased draining lymph. Although there is little doubt that most of the thoracic duct lymph was draining to the exterior, the delivery of lymphocytes to the blood by the right lymph duct and from the lymphoid tissue would be unimpaired.

Output of fat.

The amount of fat lost from the body in a chylous fistula must be considerable. No determinations of blood or lymph fat were made in Mrs. F. Munk and Rosenstein (1891) recovered 60 p.c. of fed fats from the lymph in a patient with a chylous fistula in the leg. Glenn *et al.* (1949) found in unanaesthetized dogs that the blood fat did not rise after a fatty meal when the right lymph duct had been tied and a permanent thoracic duct fistula maintained with a polythene cannula. It is probable that fat mobilized from the body stores is lost in chyle as well as a large proportion of absorbed fat, since the thoracic duct lymph in fasting dogs contains a considerable amount of fat, in greater concentration than in the blood (Rony, Mortimer and Ivy, 1932). A continued loss of weight in patients with chylous fistula even when a high protein diet is given (Crandall *et al.*, 1943) is, therefore, understandable.

T1824.

In the estimation of plasma volume by the dye method, the dilution of the dye is measured by extrapolating plasma concentrations to zero time in order to correct for dye loss from the circulation. The concentrations of dye in the plasma are followed for 10 to 30 minutes or longer after injection.

Peters (1944) drew attention to the difficulty in allowing for the return of dye to the circulation by way of the lymph when extrapolating for dye loss. Cardozo (1940–41) found that T1824 appeared in the thoracic duct lymph of fasting, anaesthetized dogs within 15 to 30 minutes after injection, and that the concentration of dye in the lymph at the end of 1 hour was approximately 30 p.c. of that in the plasma. He did not, however, calculate the amount of dye appearing in the lymph. Ferrebee, Leigh asd Berliner (1941), whose results are often quoted in support of an appreciable dye return to the circulation, found that in fasting, anaesthetized dogs, dye appeared in the thoracic duct about 15 minutes after injection and at 1 hour was 12 p.c. of the plasma concentration. Even though the lymph flow appears to have been exceedingly high, as much as 10 ml./kg./hr., calculation from their scanty data suggests that in the first hour the amount of dye collected in the lymph was of the order of 1 p.c. of the injected dose. Courtice (1943) in experiments with anaesthetized dogs and goats found that T1824 entered the thoracic duct lymph about 15 minutes after injection, and was on an average 32 p.c. of the plasma level in dogs and 19 p.c. in goats at the end of an hour. In these experiments the dye was first extracted from the lymph with n-butyl alcohol (Harington, Pochin and Squire, 1940) as it was found impossible to obtain accurate determinations otherwise. Although the concentration of dye in the thoracic duct lymph reached fairly high levels at the end of 1 hour, the actual amount of dye collected in half an hour was on an average only 0·16 p.c. of that injected in dogs and 0·07 p.c. in goats, while in 1 hour these amounts were 0·59 p.c. and 0·43 p.c. respectively. Simmonds (1950) showed that in 3 anaesthetized cats an average of 0·55 p.c. of the injected dye could be recovered from the thoracic duct in one hour.

All these experiments on animals show that in the first half-hour or hour the actual amount of dye returned is insignificant. In Mrs. F. dye first appeared in the thoracic duct in the 20 to 30 minutes' sample after injection, so that in the first half-hour practically no dye was returned by the lymph. At the end of an hour the concentration in the lymph was 12·5 p.c. of that in the

plasma, but only 0·14 p.c. of the amount of injected dye was present in all the lymph collected in the hour. The rate of lymph flow in this hour varied from 1·0 to 1·5 ml./kg./hr. It has been stated earlier that the lymph flow might be trebled by a fatty meal or by exercise. Even in these conditions it is improbable that a significant amount of dye will be returned by the lymph during the first half-hour or hour after intravenous injection of T1824. It seems, therefore, that in the estimation of blood volume by the dye method, this error is insignificant.

SUMMARY.

Lymph was collected from a woman of 50 kg. weight with a post-operative fistula of the thoracic duct in the neck.

The rate of flow of lymph varied from 53 to 78 ml./hr., the protein concentration from 2·8 to 3·7 gm./100 ml. and the lymphocytes numbered from 1,850 to 3,800/c.mm. in different samples.

During the first 20 minutes after intravenous injection of the blue dye, T1824, for determination of plasma volume, no dye appeared in the thoracic duct lymph and only 0·14 p.c. of the injected dose was recovered during the first hour after injection. This agrees with animal experiments showing that dye returning to the circulation by way of the lymph is not a source of error in plasma volume estimation.

It is probable that most of the thoracic duct lymph was escaping from the fistula. The output of fluid and protein was comparable to that in experimental animals. Although the output of lymphocytes was rather low in this case, the duration of stay of the lymphocyte in the circulation was of the same order as that found in animals.

Acknowledgment. We are grateful to Dr. M. P. Susman who very kindly allowed us to investigate this case.

REFERENCES.

Adams, W. S., Saunders, R. H. and Lawrence, J. S. (1945): Amer. J. Physiol., 144, p. 297.

Blalock, A., Robinson, C. S., Cunningham, R. S. and Gray, M. E. (1937): Arch. Surg., Chicago, 34, p. 1,049.

Cain, J. C., Grindlay, J. H., Bollman, J. L., Flock, E. V. and Mann, F. C. (1947): Surg. Gynec. Obstet., 85, p. 559.

Cameron, G. R., Courtice, F. C. and Jones, R. P. (1947): J. Path. Bact., 59, p. 425.

Campbell, W. R. and Hanna, M. I. (1937): J. biol. Chem., 119, p. 15.

Cardozo, E. L. (1940–41): Arch. Néerland. Physiol., 25, p. 410.

Courtice, F. C. (1943): J. Physiol., 102, p. 290.

Courtice, F. C. and Steinbeck, A. W. (1950): Austral. J. exp. Biol., 28, p. 161.

Crandall, L. A. Jr., Barker, S. B. and Graham, D. G. (1943): Gastroenterology, 1, p. 1,040.

Drinker, C. K. (1945): Ann. N.Y. Acad. Sci., 46, p. 807.

Drinker, C. K. and Yoffey, J. M. (1941): Lymphatics, Lymph and Lymphoid Tissue, Harvard University Press.

Ferrebee, J. N., Leigh, O. C. and Berliner, R. W. (1941): Proc. Soc. exp. Biol., N.Y., 46, p. 549.
Glenn, W. W. L., Cresson, S. L., Bauer, F. X., Goldstein, F., Hoffman, O. and Healey, J. E. Jr. (1949): Surg. Gynec. Obstet., 89, p. 200.
Harington, C. R., Pochin, E. E. and Squire, J. R. (1940): Clin. Sci. 4, p. 311.
Heidecke, J. (1906): Folia. haemat. Lpz., 3, p. 527.
Lee, F. C. (1922): J. exp. Med., 36, p. 247.
MacNab, D. S. and Scarlett, E. P. (1932): Canad. med. Assoc. J., 27, p. 29.
Munk, I. and Rosenstein, A. (1891): Quoted by Drinker and Yoffey (1941).
Peters, J. P. (1944): Physiol. Rev., 24, p. 491.
Petersen, W. F. and Hughes, T. P. (1925a): J. biol. Chem., 63, p. 179.
Petersen, W. F. and Hughes, T. P. (1925b): *Ibid.*, 66, p. 229.
Reinhardt, W. O. (1945): Proc. Soc. exp. Biol. Med., 58, p. 123.
Rony, H. R., Mortimer, B. and Ivy, A. C. (1932): J. biol. Chem., 96, p. 737.
Sanders, A. G., Florey, H. W. and Barnes, J. M. (1940): Brit. J. exp. Path., 21, p. 254.
Simmonds, W. J. (1950): Unpublished data.
Yoffey, J. M. (1935–36): J. Anat., 70, p. 507.

FATE OF INTERRUPTED NERVE-FIBRES REGENERATING INTO PARTIALLY DENERVATED MUSCLES

by H. HOFFMAN

(From the Department of Zoology, University of Melbourne).

(Accepted for publication 31st January, 1951.)

When one segmental nerve contributing to a nervous plexus is interrupted, resulting in denervation of some of the fibres of muscles innervated from the plexus, then the surviving nerve-fibres sprout new branches which penetrate into the endo-neurial sheaths of degenerated nerve-fibres, pass down to the muscle-fibres and re-innervate the degenerated end-plates (Edds, 1950; Hoffman, 1950). The sprouting may also take place from the surviving end-plates, ultraterminal processes growing towards denervated muscle-fibres, and innervating them, possibly at the site of the degenerated end-plate (Hoffman, 1950). Thus the muscle-power is restored within a short time by a process of extension of surviving motor units.

Under conditions of moderate denervation, re-innervation is apparently completed in about one month, since after this period no un-innervated muscle-fibres can be detected, and the only endoneurial sheaths unoccupied at the periphery are those leading to muscle-fibres innervated by ultraterminal processes. The somewhat slower recovery indicated by the electrophysiological studies of Hines, Wehrmacher and Thompson (1945) and van Harreveld (1945) may be due to atrophy of muscle-fibres before re-innervation, or to an atypical response to stimulation of newly-formed end-organs, as suggested by the results of Thompson, Morgan and Hines (1950).

The interesting question now arises as to the behaviour of the interrupted nerve-fibres, when they eventually regenerate down their old pathways into the muscle already re-innervated in the manner described above. It is commonly held (Cajal, 1928; Holmes and Young, 1942) that if more than one regenerating nerve-fibre enters a single endoneurial tube, all but one will fail to mature and will eventually be resorbed, so that in the end one mature nerve-fibre occupies one sheath. Thus nerve-fibres growing down the empty endoneurial sheaths of the nerve trunk (Edds, 1949, has demonstrated that no collateral sprouting occurs within the main trunks), and encountering collaterals at the periphery, might be blocked and stop growing, or break out of the sheaths and wander in the perimysial tissue. It has, moreover, been frequently asserted—Weiss (1950); Aitken (1950); Fort (1940); Elsberg (1917)—that innervated muscle-fibres cannot be further innervated ("hyperneurotised") by growing nerve-fibres, so

that the growing fibre, encountering an innervated muscle-fibre at the termination of the endoneurial tube, must again stop growing or break out and wander in the perimysium. Eruption from sheaths has actually been observed—by collateral sprouts following partial denervation, as described above, and by regenerating nerve-fibres encountering scarred and shrunken sheaths after long atrophy (Gutmann and Young, 1944).

If, for the above reasons, the muscle is really debarred from further innervation from the regenerating fibres growing into it from the interrupted nerve, then stimulation of the latter should produce no contraction of the muscles. In the present paper physiological evidence will be given, showing that such hyperneurotisation does occur, and the histological basis for it will then be described.

Materials and Methods.

Under nembutal anaesthesia, the sciatic plexus of the white rat was exposed, and L4 or L4 and 5 were interrupted. In the first series of experiments these nerves were crushed with fine, parallel-ground artery forceps, since under these conditions the endoneurial sheaths are said to remain intact, while axons are interrupted (Gutmann and Young, 1944), thus providing optimal conditions for regeneration. In the second series, the nerves were sectioned and immediately sutured with human hair, which is neither absorbed, nor productive of appreciable tissue reaction. This latter technique resulted in substantial delay of regeneration. In both series of experiments the femoral nerve was similarly interrupted at the same level, to provide some indication of the rate of regeneration. Following femoral nerve interruption, the animal extends and rotates the affected limb, only reverting to the normal position when the femoral-innervated muscles recover. Following nerve-crushing, this time was found to be about twenty days, while section and suture increased the time to about sixty days.

Some time after femoral re-innervation was apparent, the animals were anaesthetised, the sciatic plexus exposed, and the sectioned nerves stimulated. The knee was fixed, and the tendo achilles severed and attached to a clock-spring isometric lever. A concentric, bipolar electrode, supplied from a constant-voltage, square-wave stimulator and operating at 10 volts, frequency 80/sec., was used to stimulate the nerve-roots, which were severed proximally and laid on dry, paraffin wax-impregnated tissue paper to prevent current spread to other parts of the plexus. The tetanic contractions were recorded on a kymograph drum, and tension determined by comparison with weights.

After tension determinations were completed, the muscles were prepared for histological examination by the Loewit gold method. The entire nerve-plexuses, together with the sciatic nerve to the level of formation of its main branches, were removed and fixed in 10 p.c. formalin, then sectioned serially and stained by the Alzheimer-Mann-Haggqvist technique (Haggqvist, 1936) for studies on the extent of maturation of the regenerated fibres.

EXPERIMENTAL.

Physiological.

In the first series of experiments, in which L4 and the femoral nerve or L4 and 5 were crushed, vigorous contraction of gastrocnemius was obtained by stimulating L4 thirty-eight days after crushing. Stimulation of the femoral nerve caused contraction of the muscles supplied by it. In the remaining animals in this series, stimulation of L4 more than a hundred days after crushing produced large contractions.

Since the interrupted fibres regenerated rapidly, probably reaching the muscle within thirty days, it was felt that they might have arrived before the process of collateralisation was completed, and thus have found denervated muscle-fibres to innervate. A second series of experiments, involving section and suture of the nerve-roots, and resulting in substantial delay in regeneration, was therefore carried out. This should have eliminated the possibility of competition between local and centrifugal regeneration.

From 120–163 days after operation, the animals in the second series of experiments were anaesthetised, and the sectioned roots stimulated. Tetanic contraction tensions of 50–150 gm. were recorded, somewhat less than the tension obtained by stimulating the same nerve-root on the opposite (control) side of the experimental animal, or in normal animals, but representing up to 10 p.c. of the total power of the muscle, when indirectly stimulated.

These substantial contraction tensions could only be attributed to functional re-connection of the severed fibres, since the precautions adopted eliminate both reflex stimulation and current spread; but their interpretation at this stage was not apparent, since the nerve-fibres, arriving at a muscle, encountered a completely innervated system, but apparently nevertheless succeeded in establishing functional connections.

Histological.

Before presenting the histological evidence it is necessary to emphasize that macerated and teased gold-stained material presents special difficulties in regard to photographic illustration, since although the nerve-fibres can be traced for long distances, they must be followed through many planes of focus. To overcome this difficulty superimposition of photographs taken at several levels of focus has in some cases been resorted to, but it must be appreciated that photographic illustrations cannot possess the clear detail of the original microscopic preparation. Figs. 1, 3 and 4 have been thus reconstructed.

On examination of histological preparations from animals in which nerves had been crushed, evidence was readily found of the occurrence of local re-innervation, both by collateralisation, and by ultraterminal outgrowth. Scattered amongst the typical nerve-endings were found moderate numbers of end-plates supplied by two nerve-fibres, such as end-plate *a* in Fig. 3. Two fine nerve-fibres are here seen emerging from the terminal bundle to run side-by-side, twining around each other until they reach the end-plate, which both enter and innervate. The end-ramifications of the two fibres within the end-plate are not detectably separate. In Fig. 4, two mature nerve-fibres emerge from the terminal bundle, passing side-by-side until they reach and innervate end-plate *b*. Although the association between the end-ramifications of the two fibres is not as close in this case, no sharp boundary can be found between them. In no instance is this condition encountered either in normal muscle, or in partially denervated muscle if the interrupted fibres are prevented from regenerating into the muscle. The pairs of nerve-fibres maintain separate identity as far back into the terminal bundle as they can be traced, and in some cases the origin of one fibre can be traced to collateralisation by a fibre supplying another end-plate. In some cases, when the endoneurial sheath is well stained, the two fibres are seen to lie within a single sheath.

Occasionally an even more peculiar condition was encountered: two normal end-plates, each supplied by a single normal nerve-fibre, were joined across by an ultraterminal connective, as in Figs. 1 and 2. In such instances, of course, photographic illustration is exceptionally difficult, since the two end-plates often lie in different planes of focus. In Fig. 1, normal myelinated nerve-fibres *c* and *d* supply end-plates *a* and *b*, while an ultraterminal connective *ul* passes between them. End-plate *b* is probably the one which degenerated following partial denervation, and which was reconstituted by the ultraterminal process from *a*. The two parts of *b* do not seem to be completely fused. In Fig. 2, both end-plates, *x* and *y*, may be seen to-

Fig. 1.

Fig. 2.

Fig. 3.

Fig. 4.

gether with their nerve-fibres *m* and *n*; the apparent break in *n* is due to the fact that it passes out of the plane of focus; a fine connective *ul* passes across from *x* to *y* joining them.

Not all the locally reconstituted end-plates were doubly innervated, and some sheaths within the nerve-bundle were still empty. Typical ultraterminal end-plates were also seen, devoid of any other nerve-supply than the ultraterminal process, and with empty endoneurial sheaths leading to them.

Material from animals in which nerves had been sectioned and sutured yielded larger numbers of doubly innervated end-plates and cross-connected pairs, especially in those cases in which both L4 and 5 had been interrupted. It was not uncommon to find two or three doubly innervated end-plates in a single terminal spray, whilst in one animal in which L4 and 5 had been sectioned and sutured some of the terminal bundles contained nearly 50 p.c. doubly innervated end-plates. Numerous cases were also encountered of nerve-fibres approaching end-plates, twisting around the fibres innervating these end-plates, but not actually themselves innervating; whether these are sensory fibres, diverted into inappropriate endoneurial tubes and unable to connect with muscle-fibres (Gutmann, 1945; Weiss and Edds, 1945), or whether they are motor fibres not yet connected, is uncertain. Innervation of end-plates by nerve-fibres of unequal diameter was often encountered; this occurs mostly in regions of extensive collateralisation, where a great degree of denervation had been produced, and apparently is due to excessive branching, which delays maturation of the new branches. The thinner branch to a doubly innervated end-plate is accordingly interpreted as representing the collateral which reconstituted the end-plate. In one instance, from an animal in which L4 and 5 were sectioned and sutured 162 days earlier, three nerve-fibres ran in a terminal bundle; one fibre branched to supply four end-plates, from one of which grew out an ultraterminal process innervating a fifth; the remaining two nerve-fibres each innervated secondarily one of these end-plates.

In regions in which extensive denervation had occurred, chains of ultraterminals were found, with three or four end-plates linked, and with a nerve-fibre innervating the end-plate at each end. In other cases, end-plates lying close together, but innervated from different terminal bundles, were connected by an ultraterminal process. None of the ultraterminal connectives was observed to lie within endoneurial tubes, but neurolemmal cells lined them.

DESCRIPTION OF PLATE.

Fig. 1. End-plates *a* and *b* are linked across by a thick, mature ultraterminal process *ul*. End-plate *a*, apparently reconstituted from *b* is formed by the incomplete fusion of the ramifications of the two nerve-fibres supplying it. The fibre on the right is unsharp in the photograph, but is shown sharply in the inset, lower left. From an animal in which L4 and 5 were sectioned and sutured 162 days earlier. Gold, × 450. Inset × 220.

Fig. 2. End-plates *x* and *y*, on adjacent muscle-fibres, and innervated by nerve-fibres *m* and *n* are clearly seen, although there is a gap in *n* where it passes out of focus. Between the end-plates passes a fine ultraterminal connective *ul*. L4 and femoral nerve sectioned and sutured 127 days earlier. Gold, × 450.

Fig. 3. An end-plate supplied by two fine nerve-fibres, which can be traced back as separate fibres to their points of emergence from the terminal bundle. The end-ramifications of the two fibres within the end-plate are very closely intermingled. L4 and femoral nerve sectioned and sutured 128 days earlier. Gold, × 450.

Fig. 4. End-plate *b* is innervated by two mature, myelinated nerve-fibres emerging together from the terminal bundle, and passing side-by-side. The end-ramifications of the two nerve-fibres within end-plate *b* are not as close as in *a* (Fig. 3) and the end-plate appears to be formed by the partial fusion of two smaller ones. The detail of the two nerve-fibres entering the end-plate, which is not in focus in the photograph, is shown in the inset (upper right corner). From the same animal as Fig. 3. Gold, × 450, inset × 700.

It is of interest to observe that regeneration of interrupted fibres does not appear to have been complete, for there were a few unoccupied endoneurial sheaths within the nerve-trunks, while some end-plates reconstituted by collaterals or ultraterminals were not doubly innervated.

The degree of intimacy of association of the hypolemmal ramifications of the two fibres forming the end-plate is difficult to determine. In some cases the branches appeared to have fused completely to form a syncitium, in other cases they merely regulated to form symmetrical portions, close together and sharing a common sole.

Examination of serial sections of the operated nerve-plexuses indicated a degree of maturation of the regenerated nerve-fibres intermediate between the extremes of unconnected and normally connected fibres (cf. Weiss, Edds and Cavanaugh, 1945; Aitken, Sharman and Young, 1947). Maturation was substantial, but the fibres of the operated segment were detectably smaller than those of control segments, even after 160 days.

Since doubly innervated end-plates are found only in muscles into which interrupted nerve-fibres have regenerated, the suggestion comes to mind that the latter are innervating end-plates already innervated from surviving nerve-fibres within the muscle. To test this hypothesis, the sciatic plexus in animals in which L4 had been crushed forty days earlier was again exposed, and L4 crushed again. This operation was rendered difficult owing to post-operative changes in tissue relations; however, three animals were successfully operated, and were killed four days later. Animals in which L4 had been crushed forty days earlier provided controls.

Examination of the histological preparations showed an absence of doubly innervated end-plates and cross-linked pairs, although the empty endoneurial sheaths leading to ultraterminally-innervated end-plates could be observed. This result is consistent with the above hypothesis. A more satisfying demonstration would have been given by the observation of normal and partially degenerating fibres supplying the same end-plates. For technical reasons this is difficult, since degeneration of nerve-fibres is not clearly demonstrated in the rat in gold-stained material. The control material showed double innervation and ultraterminal cross-connections. Silver staining, which provides better evidence of degeneration, was not used owing to the difficulty of following individual nerve-fibres through many sections.

DISCUSSION.

Since the regenerating nerve-fibres enter a muscle which is already, in most cases, completely innervated, and nevertheless establish substantial functional connection, and since doubly innervated end-plates and cross-linked pairs have been observed, it appears reasonable to assert that the nerve-fibres, passing down their own (following nerve-crushes) or other empty endoneurial tubes, encounter the collateral fibres at the terminal ends of these tubes, pass along with them and innervate the already re-constituted end-plates. The endoneurial sheaths leading to end-plates reconstituted by ultraterminal processes are empty all the way down, and hence present no barrier to the downward passage of the regenerating fibres. The absence of double innervation and cross-linking in cases in which the regenerated nerve has again been cut further supports this assertion.

The general scheme is shown in Fig. 5. A illustrates the relationship between normal nerve- and muscle-fibres, and indicates the point of interruption of some of the nerve-fibres. B shows the degeneration of the distal portion of the severed nerve-fibre and the axonal elements of the end-plate, while the sole-plate

(s) persists. C illustrates the re-innervation of the sole by a collateral (col) from the surviving axon (b), which has passed out of its own endoneurial sheath into that of the degenerated fibre (a), and reformed the end-plate. The inset to C shows the alternative method of local re-innervation, whereby an ultraterminal process (ul) has grown out from the surviving end-plate, and reformed the degenerated one at (u.e). At the same time the interrupted axon is regenerating, throwing out regenerative buds (r.b.), and diagram D illustrates the fusion of the regenerated fibre with the end-plate reconstituted by a collateral or (in the inset) an ultraterminal process. The result is either an end-plate supplied by two nerve-fibres, or, as shown in the inset, a pair of end-plates cross-linked.

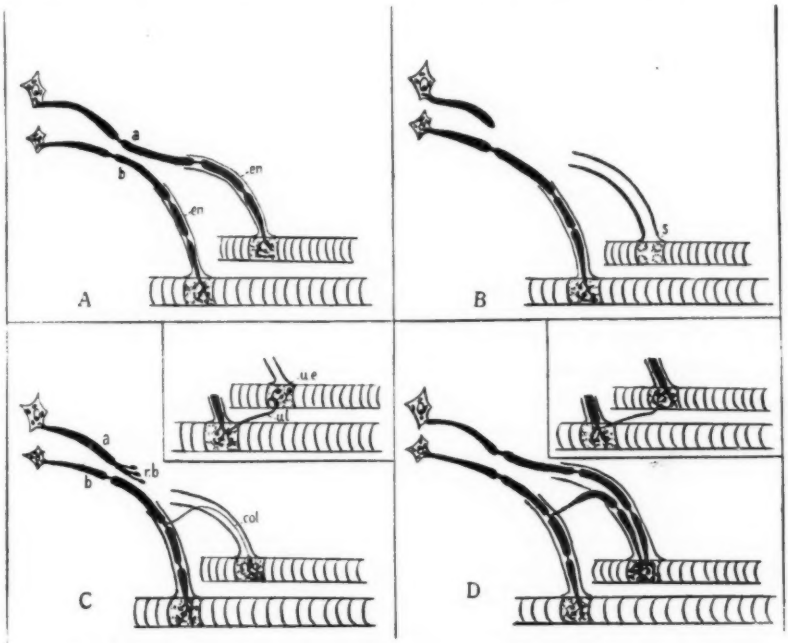

Fig. 5, illustrating the regenerative phenomena which follow partial denervation of muscles. (Description in text).

a, *b*, nerve-fibres; *en*, endoneurial sheath; *col.*, collateral branch; *r.b.*, regenerative buds at tip of axon; *s*, sole-plate; *u.e.*, ultraterminal end-plate; *u.l.* ultraterminal process.

The end-plates formed by collaterals, following partial denervation, can only lie at the sites of the original end-plates, since the collaterals are guided to the muscle-fibres by the empty endoneurial sheaths of the degenerated nerve-fibres, but ultraterminal end-plates might be formed anywhere on the muscle-fibre, since

the ultraterminal process is free in the perimysial spaces once it leaves the muscle-fibre from which it originates. The occurrence of cross-linked end-plates suggests that even ultraterminal end-plates are reformed at the sites of the old end-plates.

The nerve-fibre regenerating down an endoneurial tube occupied at the periphery by a collateral, seemingly grows alongside this fibre, fuses with its end-plate and eventually both mature, although maturation may not be complete in the case of the collateral. It is commonly considered that only one fibre can persist in each endoneurial tube, but this is at variance with the observations of Weddell, Guttmann and Gutmann (1941) who have described the passage of several cutaneous nerve-fibres along a single tube for some distance, while Gutmann and Young (1944) have described the occurrence of several nerve-fibres within a single tube, innervating a single end-plate, following regeneration after a short delay. They also described innervation of a single end-plate by two nerve-fibres coming from different directions, suggesting that they were processes of different neurones. Presumably the regenerating fibre succeeds in connecting with the end-organ, and is then no longer susceptible to resorption.

It has been assumed by nearly all authors since Harrison (1910) that the muscle-fibre resists further innervation ("hyperneurotisation") once innervated, possibly—as suggested by Fort (1940)—by a change in the penetrability of the sarcolemma, and very little reliable evidence is available in favour of the normal occurrence of multiple-innervated muscle-fibres in mammals (Denny-Brown and Pennybacker, 1938) except for the panniculus carnosus muscle of the hedge-hog (Garven, 1925). However, Hoffman (1951) has presented evidence in favour of the possibility of hyperneurotising normal muscle-fibres by means of direct nerve-implantation, and the results presented above seem to show that the muscle-fibre can be bisegmentally hyperneurotised at the end-plate by a nerve-fibre growing down the endoneurial tube, and that this innervation is functional. As has been mentioned, Gutmann and Young (1944) have described one such case in classical re-innervation, and Aitken (1950) has described what he considered might be a bi-neuronally innervated end-organ formed by nerve-fibres implanted in denervated muscle. The readiness with which hyperneurotisation is accomplished under these conditions emphasizes the important rôle of endoneurial guides in re-innervation.

In one case described previously (Hoffman, 1950), doubly innervated end-plates were obtained by chemically induced outgrowth of ultraterminal processes which, in two instances, fused with the hypolemmal ramifications of other end-plates, presenting an appearance comparable with the cross-linked end-plates described above.

Whether, in the case of doubly-innervated end-plates, the end-ramifications are confluent, is not clear; certainly, with gold-chloride staining, there is often

no detectable boundary between them. On the other hand, degeneration experiments indicate that they behave as separate trophic units, degeneration extending down one fibre until its processes disintegrate.

SUMMARY.

Interrupted nerve-fibres, regenerating down endoneurial pathways into partially denervated muscles, fuse with end-plates which have been reconstituted by the sprouting of surviving nerve-fibres. Doubly-innervated end-plates, and ultraterminally-linked pairs of end-plates are formed, and these new pathways are functional. Hyperneurotisation is readily achieved under these conditions, and degeneration experiments suggest that the neuronal processes, however intimately associated within the end-plates, behave as trophic units.

Acknowledgments. I wish to thank Prof. S. Sunderland for his valuable advice, and also Mr. E. Matthaei for his efforts in the photographic illustration of this paper.

REFERENCES.

Aitken, J. T. (1950): J. Anat., 84, p. 38.
Aitken, J. T., Sharman, M. and Young, J. Z. (1947): *Ibid.*, 81, p. 1.
Cajal, Ramon y. (1928): Degeneration and Regeneration of the Nervous System. O.U.P.
Denny-Brown, D. and Pennybacker, J. R. (1938): Brain, 68, p. 311.
Edds, MacV. Jnr. (1949): J. exp. Zool., 111, p. 211.
Edds, MacV. Jnr. (1950): *Ibid.*, 113, p. 517.
Elsberg, C. A. (1917): Science, 45, p. 318.
Fort, W. B. (1940): An Experimental Study of the Factors Involved in the Establishment of Neuromuscular Connections, Chicago Univ.
Garven, H. S. D. (1925): Brain, 48, p. 380.
Gutmann, E. (1945): J. Anat., 79, p. 1.
Gutmann, E. and Young, J. Z. (1944): *Ibid.*, 78, p. 15.
Haggqvist, G. (1936): Z. mikr. Anat. Forsch., 39, p. 1.
van Harreveld, A. (1945): Amer. J. Physiol., 144, p. 477.
Harrison, R. G. (1910): J. exp. Zool., 9, p. 787.
Hines, H. M., Wehrmacher, W. H. and Thompson, J. D. (1945): Amer. J. Physiol., 145, p. 48.
Hoffman, H. (1950): Austral. J. exp. Biol., 28, p. 383.
Hoffman, H. (1951): In the Press.
Holmes, W. and Young, J. Z. (1942): J. Anat. 77, p. 63.
Thompson, J. D., Morgan, J. A. and Hines, H. M. (1950): Amer. J. Physiol., 161, p. 142.
Weddell, C., Guttmann, L. and Gutmann, E. (1941): J. Neurol. Psychiat., 4, p. 206.
Weiss, P. (1950): J. exp. Zool., 113, p. 397.
Weiss, P. and Edds, MacV. Jnr. (1945): J. Neurophysiol., 8, p. 173.
Weiss, P., Edds, MacV. Jnr. and Cavanaugh, M. (1945): Anat. Rec., 92, p. 215.

THE ISOLATION AND CHARACTERISTICS OF BACTERIOPHAGES FROM STAPHYLOCOCCI OF ANIMAL ORIGIN

by SIBELY J. McLEAN.

(From the Department of Bacteriology, University of Adelaide).

(Accepted for publication 7th March, 1951.)

The main investigations concerning the phenomenon of lysogenicity in staphylococci and the relationships of groups of phages have been carried out with groups of cultures and phages which were mainly of human origin and which included the standard typing phages of Wilson and Atkinson (1945) and their propagating strains (Williams Smith, 1948, a, b; Rountree, 1949 a, b).

Staphylococcal phages can be readily isolated from lysogenic strains by the cross-culture method (Fisk, 1942; Wilson and Atkinson, 1945; Rountree, 1947, 1949 a, b; Williams Smith, 1948 a, b), and many authors have described the isolation of phages from coagulase-positive strains of human origin. Williams Smith (1948a) investigated a number of type 42D strains of bovine origin by the cross culture method and isolated phages from many of them. The only other reference to the isolation of phages from strains of animal origin is that of Slanetz and Jawetz (1941) who isolated the phages by the conventional phage technique using filtrates from the milk from cows suffering from mastitis.

As a number of bovine strains of staphylococci was available the present investigation was undertaken to determine if phages could be isolated by the cross-culture method from strains of staphylococci of animal origin as readily as from strains of human origin, and to study the cross-reactions, biological characteristics and serological relationships of the phages.

Materials and Methods.

Thirty-six strains of staphylococci were investigated; twenty-eight of these came from cases of bovine mastitis, five from cases of ovine mastitis, one from the spleen of a rat, one from a boil on a cat and one from a skin infection of a bull-dog. All these strains produced either α, β, or $\alpha\,\beta$ haemolysis when tested by the spot plate method of Bryce and Rountree (1936), and all strains except two were coagulase-positive by the slide test method of Cadness-Graves *et al.* (1943).

The strains of staphylococci were kept on nutrient agar in the cold and were subcultured every five weeks.

Isolation of the phages.

The medium used for all phage work was 0·5 p.c. nutrient agar. This was made from powdered agar which gave a gel of consistency comparable with that of 1·2 p.c. shredded agar. The technique used for the isolation and purification of the phages was that described by Wilson and Atkinson (1945) and modified by Williams Smith (1948a), with the exception that the original streak method of Fisk (1942) was used instead of spreading the basal strain over the plates as in the method of Wilson and Atkinson. The plates were incubated at 37° C. for approximately five hours then left overnight at a temperature between 9–13° C.

Phages were also isolated from filtrates of lysogenic strains by the method of Rountree (1949a). Two drops of culture were added to about 8 ml. of nutrient broth which was then incubated for 4–5 hours. The culture was then centrifuged at 2,500 r.p.m. for 15 minutes and filtered through a gradocol membrane of an A.P.D. of 600 mμ. The filtrate was tested against a series of known sensitive strains.

Propagation of the phages.

This was carried out by a broth method. One loopful of an overnight broth culture of the propagating strain was added to about 5 ml. of nutrient broth and after 1–2 hours' incubation at 37° C., 0·6 ml. of the phage preparation was added and the broth was then incubated until lysis took place. If lysis did not take place within 4 hours, the preparation was left at 13° C. overnight.

Phage preparations were filtered through gradocol membranes of 600 mμ A.P.D. and stored in the refrigerator. The phages were designated according to the method of Wilson and Atkinson by the organism from which they were derived and by the strain on which they were propagated.

Titration of the phages.

Titration of phage preparations was carried out by the method of Craigie and Yen (1938) used for the typing of typhoid bacilli with Vi phages, with the exception that the temperature and time of incubation were those used above for staphylococcal phages. The critical test dilution (C.T.D.) was recorded as the highest dilution giving confluent lysis.

Phage typing tests were set up in the same way as these titrations.

Cross-resistance tests.

These were carried out according to a modification of the original method of Bail (1923). Resistant cultures were prepared according to the method of Williams Smith (1948a) and were designated by the number of the culture, followed by the number of the phage in brackets, e.g. the resistant culture of 7 to the phage 17/7 was 7 (17/7).

Determination of plaque size.

This was carried out on 0·5 p.c. nutrient agar to which had been added 0·01 p.c. Difco yeast extract and 5 p.c. Fildes' peptic digest of blood. The phage preparations were diluted in sterile nutrient broth to give between 50–200 plaques, then were plated out with the propagating strain and incubated overnight at 37° C. The size of 30 plaques was measured using a colony microscope with a micrometer eyepiece.

Ability to lyse in the presence of citrate.

Citrate-sensitivity tests were carried out according to the method of Burnet (1933b). 0·5 p.c. nutrient agar plates were poured containing in 20 ml. of medium 0·5, 1·0, 2·0 and 3·0 ml. of a 10 p.c. solution of sodium citrate. The propagating strain was spread over a 1·5 cm. area on the plate and a loopful of the undiluted phage filtrate placed on the area. The plates were incubated at 37° C. for 4–5 hours and left at 9–13° C. overnight.

Serology.

Antisera were prepared in rabbits by the injection of gradocol filtrates of lysed broth cultures. The course of injections was 1·0 ml., 1·5 ml., 1·75 ml. and 2·0 ml. given at weekly intervals into the marginal vein of the ear, the rabbit being bled on the 8th, 10th and 12th days after the last injection.

Antisera were prepared against phages 7 and 44A of the standard typing collection as these phages represent two different serological groups (personal communication Dr. V. D. Allison; Rountree, 1949b).

Neutralization tests were carried out by the method of Burnet (1933a) with the exception that the counting of plaques was done by a pour plate method based on that of Yen (1935) instead of by the surface method.

Tenfold dilutions of serum were made in nutrient broth and an equal volume of diluted phage added to each serum dilution. The phage was diluted to give a countable number of plaques (50–200) when plated by the pour plate method. The phage-serum mixtures were incubated at 37° C. for 4 hours and were then plated in duplicate by the pour plate method.

Pour plates were prepared as follows: one oz. McCartney bottles containing 8 ml. of melted 0·5 p.c. nutrient agar were placed in a water bath at 48° C. and 0·2 ml. of a 24 hour culture of the propagating strain of *Staphylococcus* added. The bottles were removed from the bath one at a time; 0·8 ml. of the incubated phage-serum mixture was added, the agar was shaken carefully to ensure even distribution of the phage particles then the mixture was poured immediately into small Petri plates, 8 cm. diameter, care being taken to avoid bubbles in the agar. After incubation overnight at 37° C. the number of plaques was counted. The titre of the serum was expressed as the reciprocal of that dilution which gave approximately 80 p.c. reduction in the plaque count as compared with that given by controls consisting of phage incubated with normal rabbit serum or broth.

TABLE 1.

Results of the cross-culture tests showing lysogenic and susceptible strains.

Susceptible strains.	Lysogenic strain 1	5	9	11	12	13	17	18	24	25	27	29	30	31	32	35
	7	4	1	1	1	1	1	1	1	1	1	1	1	1	1	1
	8						7	7			8	7		7		
	10						8	8			9	8		8		
	17						10	10			10	9		9		
	18						21	21			11	10		10		
	21						29	29			12	11		11		
	29						31	31			13	12		12		
	31										17	13		13		
											18	17		17		
											20	18		18		
											21	20		20		
											24	21		21		
											25	24		24		
											30	25		25		
											32	30		30		
											34	32		32		
											35	34		34		
												35		35		

RESULTS.

The cross-culture tests showed that many of the strains were lysogenic and that there was a large number of susceptible strains (Table 1.). The lysogenic and susceptible strains fell into groups, each lysogenic strain in the group having the same susceptible strains. One lysogenic strain was selected from each group and was propagated on each of the susceptible strains. The phages carried by the strains 1, 5, 9, 11, 12, 13, 17, 24, 25, 29, 30, 32 and 35 were purified and propagated on all the susceptible strains listed in Table 1.

The nine phages 9/1, 11/1, 12/1, 13/1, 24/1, 25/1, 30/1, 32/1 and 35/1 when diluted to the C.T.D. all completely lysed strain 1 and so appeared to be identical. This was proved by cross-resistance tests which showed that none of the phages had any action on the resistant strains of 1 prepared against the other eight phages.

The lysogenic strains 1, 17 and 29 had from seven to eighteen susceptible strains, so the phages from these three strains, propagated on each of the susceptible strains, were investigated by cross-resistance tests to determine if they were identical, or if each lysogenic strain was carrying several phages.

The phage from strain 1 was propagated on the eight susceptible strains listed in Table 1. All eight strains were tested with the phages diluted to the C.T.D. and all eight strains were completely lysed by each of the phages. Resistant strains of each organism were also tested with all the phages. If the phages were identical none of them should have had any action on the resistant strains prepared against the other seven phages. The results from the cross-resistance tests showed that the phages were not all identical but fell into three groups (Table 2). Phages 1/7, 1/8, 1/10 and 1/21 had no action on the resistant strains, 1/29 and 1/31 had some action on four resistant strains, while 1/17 and 1/18 lysed all the resistant strains except 17 (1/17) and 18 (1/18).

TABLE 2.

Lytic reactions of the phages with their resistant strains.

Phage	Resistant strains							
	7(1/7)	8(1/8)	10(1/10)	17(1/17)	18(1/18)	21(1/21)	29(1/29)	31(1/31)
1/7	—	—	—	—	—	—	—	—
1/8	—	—	—	—	—	—	—	—
1/10	—	—	—	—	—	—	—	—
1/17	CL	CL	CL	—	—	CL	CL	CL
1/18	CL	CL	CL	—	—	CL	CL	CL
1/21	—	—	—	—	—	—	—	—
1/29	+	+	+	—	—	+	—	—
1/31	±	±	±	—	—	±	—	—

Phage	Resistant strains						
	1(17/1)	7(17/7)	8(17/8)	10(17/10)	21(17/21)	29(17/29)	31(17/31)
17/1	—	++	++	++	++	—	—
17/7	—	—	—	—	—	—	—
17/8	—	—	—	—	—	—	—
17/10	—	—	—	—	—	—	—
17/21	—	—	—	—	—	—	—
17/29	+	—	+	—	—	—	—
17/31	+	—	+	—	—	—	—

TABLE 2 (Continued).

Phage	Resistant strains									
	1 (29/1)	7 (29/7)	8 (29/8)	9 (29/9)	10 (29/10)	11 (29/11)	12 (29/12)	13 (29/13)	17 (29/17)	18 (29/18)
29/1	—	±	±	—	±	±	—	—	—	—
29/7	—	—	—	—	+	+	—	—	—	—
29/8	—	—	—	—	±	±	—	—	—	—
29/9	—	—	—	—	±	±	—	—	—	—
29/10	—	—	—	—	—	—	—	—	—	—
29/11	—	—	±	—	±	—	—	—	—	—
29/12	—	—	—	—	+	±	—	—	—	—
29/13	—	—	—	—	+	±	—	—	—	—
29/17	+	+	±	—	±	—	—	—	—	—
29/18	++	++	++	—	++	—	—	—	—	—
29/20	—	—	—	—	—	—	—	—	—	—
29/21	—	—	—	—	—	—	—	—	—	—
29/24	—	—	—	—	±	±	—	—	—	—
29/25	—	—	—	—	±	±	—	—	—	—
29/30	—	—	—	—	—	—	—	—	—	—
29/32	—	—	—	—	—	—	—	—	—	—
29/34	—	—	—	—	—	—	—	—	—	—
29/35	—	—	—	—	—	—	—	—	—	—

Phage	Resistant strains							
	20 (29/20)	21 (29/21)	24 (29/24)	25 (29/25)	30 (29/30)	32 (29/32)	34 (29/34)	35 (29/35)
29/1	±	—	—	—	±	—	—	—
29/7	—	—	—	—	+	+	+	+
29/8	—	—	—	—	—	+	—	+
29/9	—	—	—	—	—	+	—	+
29/10	—	—	—	—	—	—	—	—
29/11	—	—	—	—	—	—	—	—
29/12	—	—	—	—	+	+	+	+
29/13	—	—	—	—	+	+	+	+
29/17	+	—	—	—	+	+	—	+
29/18	++	—	—	—	++	—	—	—
29/20	—	—	—	—	—	—	—	—
29/21	—	—	—	—	—	—	—	—
29/24	—	—	—	—	+	+	+	+
29/25	—	—	—	—	—	+	+	+
29/30	—	—	—	—	—	+	+	+
29/32	—	—	—	—	—	—	—	—
29/34	—	—	—	—	—	—	—	—
29/35	—	—	—	—	—	—	—	—

CL = Confluent lysis; ++ = Semi—confluent lysis; + = Discrete plaques; ± = Less than 20 plaques.

Reference to Table 2 shows that similar results were obtained with the phages from the strains 17 and 29. In both cases all the phages when diluted to the CTD lysed all the susceptible strains but cross-resistance tests showed that the phages were not identical but fell into several groups.

The phages from each strain appeared to be identical in their lytic reactions but differed in their cross-resistance tests. All the reactions in the cross-resistance tests proved to be due to the presence of phage from the lysogenic propagating strains and not the original phage. Gradocol filtrates were prepared from several lysogenic strains, namely 1, 17 and 29, and when tested on three sets of resistant strains they gave reaction patterns which explained the results from the cross-resistance tests (Table 3). The filtrates from 1, 17 and 29 did not act on all the resistant strains as would be expected e.g. the filtrate from 29 did not lyse 17 (1/17) although the phage from 29 could be propagated on 17. This could have been due to the fact that strain 17, when made resistant to the phage from 1, also became resistant to the phage from 29.

TABLE 3.

Lytic reactions of filtrates of lysogenic strains on certain resistant strains.

Filtrate of 5 hour culture of lysogenic strain	Resistant strains							
	7 (1/7)	8 (1/8)	10 (1/10)	17 (1/17)	18 (1/18)	21 (1/21)	29 (1/29)	31 (1/31)
1	—	—	—	—	—	—	—	—
17	+	+	+	—	—	+	+	+
29	++	++	++	—	—	++	—	—

Filtrate of 5 hour culture of lysogenic strain	Resistant strains						
	1 (17/1)	7 (17/7)	8 (17/8)	10 (17/10)	21 (17/21)	29 (17/29)	31 (17/31)
1	—	+	+	+	+	—	—
17	—	—	—	—	—	—	—
29	+	—	±	—	—	—	—

Filtrate of 5 hour culture of lysogenic strain	Resistant strains								
	1 (29/1)	7 (29/7)	8 (29/8)	9 (29/9)	10 (29/10)	11 (29/11)	12 (29/12)	13 (29/13)	17 (29/17)
1	—	+	+	—	++	—	—	—	+
17	++	+	+	—	++	—	—	—	—
29	—	—	—	—	—	—	—	—	—

Filtrate of 5 hour culture of lysogenic strain	Resistant strains								
	18 (29/18)	20 (29/20)	21 (29/21)	24 (29/24)	25 (29/25)	30 (29/30)	32 (29/32)	34 (29/34)	35 (29/35)
1	+	+	—	—	—	+	—	—	—
17	—	+	—	—	—	+	—	—	—
29	—	—	—	—	—	—	—	—	—

++ = Semi-confluent lysis; + = Discrete plaques; ± = Less than 20 plaques.

The results recorded in Tables 2 and 3 brought out two points of interest. Firstly, the actions of the phages were frequently complicated by trace reactions due to the phage from the lysogenic propagating strains. Secondly, the lysogenic strains 1, 17 and 29 each carried only one phage. When these phages were propagated on any of the non-lysogenic strains, 7, 8, 10 or 21 the typing reactions obtained were those of the phages themselves. However, when the phages were propagated on any of the lysogenic strains such as 1, 17 or 29 trace reactions appeared due to the presence of phage from these propagating strains. These trace reactions can be seen in Table 4; for example 1/7 gives the reactions of the phage carried by 1 whereas the action of 1/29 is enlarged by the phage from 29.

TABLE 4.

Lytic action of the phages on the complete set of animal strains.

Phage	Staphylococcal strain																	
	1	2	3	4	5	6	7	8	9	10	11	12	13	14	15	16	17	18
1/7	—	—	—	++	++	—	CL	CL	—	CL	—	—	—	—	±	—	CL	CL
1/17	±	—	—	—	+	—	CL	CL	—	CL	—	—	—	—	±	—	CL	CL
1/29	+	—	—	—	—	—	CL	CL	++	CL	+	+	+	—	±	—	CL	CL
17/1	CL	—	—	—	+	—	CL	CL	—	CL	—	—	—	—	+	—	++	++
17/7	CL	—	—	—	—	—	CL	CL	—	CL	—	—	—	—	—	—	—	—
17/29	CL	—	—	—	—	—	CL	CL	+	CL	+	+	+	—	—	—	+	+
29/1	CL	—	—	—	—	—	CL	CL	CL	CL	CL	CL	CL	—	—	—	CL	CL
29/7	CL	—	—	—	—	—	CL	CL	CL	CL	CL	CL	CL	—	—	—	CL	CL
29/17	CL	—	—	—	—	—	CL	CL	CL	CL	CL	CL	CL	—	—	—	CL	CL
11/1	CL	—	—	—	—	—	+	+	—	+	—	—	—	—	—	—	±	±
5/4	—	±	—	CL	+	CL	—	+	±	±	—	—	—	—	—	—	—	—

Phage	Staphylococcal strain																	
	19	20	21	22	23	24	25	26	27	28	29	30	31	32	33	34	35	36
1/7	+	CL	CL	—	—	—	—	—	—	—	CL	—	CL	—	—	+	±	—
1/17	+	CL	CL	—	—	—	—	—	—	—	CL	—	CL	—	—	—	—	—
1/29	+	CL	CL	—	—	+	+	—	±	—	CL	+	CL	++	—	+	+	—
17/1	+	CL	CL	—	—	—	—	±	—	—	CL	—	CL	—	—	—	—	±
17/7	—	CL	CL	—	—	—	—	—	—	—	CL	—	CL	—	—	—	+	—
17/29	—	CL	CL	—	—	+	+	—	—	—	CL	+	CL	+	—	+	+	—
29/1	—	CL	CL	—	—	CL	CL	—	—	—	—	CL	—	CL	—	CL	CL	—
29/7	—	CL	CL	—	—	CL	CL	—	—	—	—	CL	—	CL	+	CL	CL	—
29/17	—	CL	CL	—	—	CL	CL	—	—	—	++	CL	++	CL	++	CL	CL	—
11/1	—	+	+	—	—	—	—	—	—	—	+	—	+	—	—	±	—	—
5/4	—	+	±	—	—	—	—	—	—	—	±	—	±	—	—	—	—	—

CL = Confluent lysis. ++ = Semi-confluent plaques. + = Discrete plaques. ± = Less than 20 plaques.

Investigations of plaque size, ability to lyse in the presence of citrate, and serology were carried out on a representative set of the phages namely: 1/7, 1/17, 1/29, 17/1, 17/7, 17/29, 29/1, 29/7, 29/17, 11/1 and 5/4. Phages 7 and 44A of the standard typing collection were included for comparison.

Measurements of plaque size showed that all the phages had plaques of approximately the same size range (Table 5).

TABLE 5.

Plaque sizes of staphylococcal phages.

Phage	Plaque size (mm.)	
	Range	Mean
1/7	0·18—0·74	0·42
1/17	0·18—0·59	0·41
1/29	0·26—0·85	0·42
17/1	0·14—0·7	0·45
17/7	0·07—0·52	0·24
17/29	0·07—0·77	0·4
29/1	0·37—1·65	0·99
29/7	0·18—0·77	0·51
29/17	0·12—0·96	0·45
11/1	0·15—0·92	0·68
5/4	0·07—0·7	0·31
7	0·18—0·92	0·41
44A	0·14—1·26	0·46

TABLE 6.

Ability to lyse in the presence of citrate.

Lowest concentration of citrate showing complete inhibition of lysis	Phage
0·25 p.c.	29/1, 29/7, 29/17, 7
0·5 p.c.	—
1·0 p.c.	17/7
1·5 p.c.	1/7, 1/29, 17/29, 5/4
Unaffected by 1·5 p.c.	11/1, 1/17, 17/1, 44A

TABLE 7.

Phage neutralization tests.

Phage	Antisera against	
	44A	7
44A	10,000	—
7	—	10,000
1/7	—	10,000
1/17	—	10,000
1/29	—	1,000
17/1	—	10,000
17/7	—	1,000
17/29	—	1,000
29/1	—	—
29/7	—	—
29/17	—	—
11/1	—	10,000
5/4	—	10,000

Results are expressed as the reciprocals of the serum dilutions neutralising 80 p.c. of the phage.

Phages 29/1, 29/7 and 29/17 were completely inhibited by 0·25 p.c. citrate while 1/17, 1/29, 17/1 and 11/1 were not affected by 1·5 p.c. citrate (Table 6).

All the phages except 29/1, 29/7 and 29/17 were neutralized by antiserum prepared against phage 7. These three phages were not neutralized by the antiserum prepared against phage 7 r phage 44A (Table 7).

Thus in their general characteristics all the phages appeared to be similar except the phage from strain 29. The identical phages 29/1, 29/7 and 29/17, were inhibited by 0·25 p.c. citrate whereas the other phages were not and these three phages did not belong to the same serological group as the other phages.

DISCUSSION.

The twenty-one lysogenic and susceptible bovine strains and their phages formed an interesting group as they appeared to be closely inter-related and a remarkable number of lytic combinations was obtained with these twenty-one strains. Most of the strains came from herds near Adelaide so there might have been cross-infection of the animals with the same strain. Williams Smith (1948 b) reported that when two strains of staphylococci, one of which was lysogenic to the other, were grown together in broth the susceptible strain underwent a change in phage type and he had evidence that this change of type could also take place in the field. In the present series some of the phage types may have originated by this process.

However, strain 1 which figured largely in the lytic combinations came from Tasmania, strain 8 from Western Australia and strain 25 from Queensland.

The typing reactions of the phages isolated in this study confirmed the report of Williams Smith (1948 a, b) that acquired phage resistance might be responsible for the classification of many originally identical strains as different phage types. For example, two strains that were identical apart from the fact that one of them had acquired a resistance to a phage might be classified as two different phage types. This can be seen in Table 8 which records the lytic action of phage 44A and the phages from 1, 17 and 29 on twenty staphylococci. All these strains were lysed by phage 44A but they were not all lysed by the other three phages. The strains 1, 9, 11, 12, 13, 17, 18, 24, 25, 29, 30, 31, 32 and 35 were lysogenic and were resistant to certain of the phages. These strains all belonged to phage type 44A yet when they were typed with the phages from 1, 17 and 29 they appeared to fall into several groups due to acquired phage resistance.

Williams Smith found that lysogenic strains were insusceptible to the phages they carried. Rountree (1949 a) showed that the possession of lysogenicity did not render staphylococci resistant to the lytic action of bacteriophages. In several cases she found that after passage of a phage through a sensitive strain it appeared to be modified in infectivity so that the previously

resistant cells of the parent became sensitive. Several of her lysogenic strains carried as many as five distinct bacteriophages, but only one, or at the most three, of the phages from any lysogenic strain could be modified to act on that strain.

TABLE 8.

Lytic action of four phages on twenty-one bovine strains of staphylococci.

Phage	Staphylococcal strain									
	1	7	8	9	10	11	12	13	17	18
44A	CL	CL	CL	CL	CL	CL	CL	CL	CL	CL
1	—	CL	CL	—	CL	—	—	—	CL	CL
17	CL	CL	CL	—	CL	—	—	—	—	—
29	CL	CL	CL	CL	CL	CL	CL	CL	CL	CL

Phage	Staphylococcal strain									
	20	21	24	25	29	30	31	32	34	35
44A	CL	CL	CL	CL	CL	CL	CL	CL	CL	CL
1	CL	CL	—	—	CL	—	CL	—	—	—
17	CL	CL	—	—	CL	—	CL	—	—	—
29	CL	CL	CL	CL	—	CL	—	CL	CL	CL

CL = Confluent lysis.

In the present study no lysogenic strain was found which carried more than one phage. The lysogenic strains were still susceptible to the lytic action of certain of the phages but they were never susceptible to the action of the phages they carried.

There were other points of interest that emerged from the results of this study. The phage 5/4 was an ovine phage and completely lysed only two strains, 4 and 6, which were both of ovine origin. Neutralization tests showed that this phage belonged to serological group A. Rountree (1949 b) reported that three phages lysing staphylococci pathogenic to sheep fell into a separate serological group—Group C.

Macdonald (1946) showed that in Norfolk the majority of milk strains of staphylococci and those isolated from clinical cases of bovine mastitis belonged to phage type 42D. In the present series twenty out of twenty-eight bovine strains belonged to type 44A.

SUMMARY.

Bacteriophages can be readily isolated from strains of staphylococci of animal origin by the cross-culture method and in some cases they can be detected in filtrates of young broth cultures of the lysogenic strains.

From a set of 36 strains 85 lytic combinations were detected and 43 phages were isolated and purified. Cross-resistance tests showed that many of these phages were identical, only 5 different phages being present. All the phages except that isolated from one strain belonged to serological group A and were not sensitive to 0·25 p.c. citrate.

No lysogenic strain was found which carried more than one phage.

When tested on the original 36 strains the phages gave a reaction pattern which was affected by the lysogenicity of certain of the strains.

Acknowledgments. My thanks are due to Mr. M. F. Pulsford, Veterinary Pathologist, Institute of Medical and Veterinary Science, Adelaide, and to Mr. R. V. S. Bain, former Veterinary Pathologist of the same Institute for supplying many of the strains.

Mr. G. C. Simmons, Animal Health Laboratory, Yeerongpilly, Queensland, kindly supplied a strain from a case of bovine mastitis. Dr. V. D. Allison, Central Public Health Laboratory, Colindale, London, kindly supplied a set of the standard typing phages and their propagating strains.

My thanks are also due to Miss Nancy Atkinson for her help in the preparation of the manuscript.

REFERENCES.

Bail, O. (1923): Z. Immunforsch., 38, p. 57.
Bryce, L. M. and Rountree, P. M. (1936): J. Path. Bact., 43, p. 173.
Burnet, F. M. (1933a): *Ibid.*, 36, p. 307.
Burnet, F. M. (1933b): *Ibid.*, 37, p. 179.
Cadness-Graves, B., Williams, R., Harper, G. J. and Miles, A. A. (1943): Lancet, 244, p. 736.
Craigie, J. and Yen, C. H. (1938): Canad. Pub. Health J., 29, p. 448.
Fisk, R. T. (1942): J. infect. Dis., 71, p. 153.
Macdonald, A. (1946): Mon. Bull. Min. Health, Lond., 5, p. 230.
Rountree, P. M. (1947): Austral. J. exp. Biol., 25, p. 9.
Rountree, P. M. (1949a): J. gen. Microbiol., 3, p. 153.
Rountree, P. M. (1949b): *Ibid.*, 3, p. 164.
Slanetz, W. and Jawetz, E. (1941): J. Bact., 41, p. 447.
Williams Smith, H. (1948a): J. Hyg. Camb., 46, p. 74.
Williams Smith, H. (1948b): *Ibid.*, 46, p. 82.
Wilson, G. S. and Atkinson, J. D. (1945): Lancet 248, p. 647.
Yen, A. C. H. (1935): Proc. Soc. exp. Biol., 32, p. 1,006.

THE EXTRACTION OF LIVER ANTICOAGULANT OTHER THAN HEPARIN

by F. E. BINET, E. R. TRETHEWIE AND FAY M. GAFFNEY[1]

(From the Institute of Medical and Veterinary Science, Adelaide, the Department of Experimental Medicine, University of Adelaide, and the Department of Physiology, University of Melbourne).

(Accepted for publication 11th January, 1951.)

It has been suggested previously (Trethewie, 1945b, and Trethewie, Cleland and Pengelley, 1947) that the anti-coagulant released from the liver during a perfusion with diluted heparinized blood might be similar to the anti-coagulant extracted from tissues by De Sütö-Nagy (1944). De Sütö-Nagy has shown that the sphingomyelin which he extracted, formed a complex with albumin and that this complex acted as an anti-coagulant. In the earlier paper, (Trethewie, 1945b) it was found that a protein fraction liberated from the perfused liver possessed anti-coagulant properties, and it was suggested that the anti-coagulant might either be a protein or that a complex of protein and lipid was responsible for anti-coagulability. In the second paper (Trethewie, Cleland and Pengelley, 1947) it was suggested that the active substance was probably a sphingomyelin, on the basis of its solubilities. Trethewie and Day (1949) treated the partially purified anti-coagulant by the method of Thannhauser, Benotti and Boncoddo (1946) and demonstrated that the active ingredient was not hydrolecithin, and that probably it was not sphingomyelin unless its solubility was altered by other constituents. In this paper an attempt has been made to obtain the anti-coagulant in the liver in as pure a form as possible and to identify it. This is considered important since this anticoagulant is a "physiological" one and there are certain drawbacks to anti-coagulants at present in use, clinically. Further, it is considered likely that this substance can prevent clotting *in vivo*.

EXPERIMENTAL.

Under i.v. pentothal anaesthesia, the livers of three dogs were perfused with Tyrode until all visible blood was removed. The connective tissue was cut away and the livers cut up and minced. 555 gm. wet weight of tissue paste was obtained and it was dried in the following manner. The tissue paste was allowed to stand with 800 ml. acetone for three periods of 24 hours and then with 800 ml. absolute acetone at 4° C. twice for the same time and finally with 800 ml. absolute acetone for two periods of 24 hours at room temperature. The acetone-dehydrated tissue then was ready for extracting.

[1] This work was aided by a grant from the National Health and Medical Research Council of Australia.

First extractions: The tissue paste was extracted twice with 800 ml. absolute ether at 4° C. for 24 hours, and then with 2 litres boiling ether for 12 hours in a "Soxhlet" apparatus. The material was dried and ground in a mill and pulverized with powdered porous glass.

Second extractions: The product thus obtained was extracted in a "Soxhlet" apparatus with 2 litres boiling acetone for 12 hours and dried.

Third extractions: The material was then extracted with 500 ml. 99 p.c. alcohol for two periods of 24 hours, 500 ml. absolute alcohol at 4° C. twice, 500 ml. absolute alcohol at 37° C. for 24 hours and finally with 1 litre boiling absolute alcohol for three periods of 30 minutes. It was then Soxhlet-extracted with boiling alcohol for 12 hours and dried.

Fourth extractions: This resultant tissue powder was treated with 500 ml. of a mixture of chloroform and methanol (3:1 by volume) for periods of 24 hours at 4° C., 24 hours at 37° C., and 30 minutes at boiling point. It then was extracted with 2 litres of 3:1 chloroform-methanol mixture for 12 hours in a "Soxhlet" apparatus and dried. The powder so obtained weighed 100 gm.

Subsidiary extractions: 20 gm. of this powder were mixed thoroughly with 275 mg. heparin (Boots) and the mixture was Soxhlet-extracted with 500 ml. absolute acetone, 500 ml. ether, 500 ml. alcohol and finally the same volume of 3:1 chloroform-methanol mixture.

All the extracts from the extractions above were concentrated *in vacuo*.

Anticoagulant activity.

This was estimated by considering Quick's prothrombin time, using rabbits' brain. Ca-free Tyrode was used for dilutions. The effect of the solution on the prothrombin time was matched with heparin always, the amount of anticoagulant substance which produced the same lengthening of the prothrombin time as a certain amount of heparin being determined on a blood sample.

Drying in vacuo minute amounts of material.

This was carried out in an apparatus designed as described in the Addendum.

Findings.

The anticoagulant activity of all extracts except those in acetone and alcohol was negligible. The extracts obtained from the 3:1 chloroform-methanol mixture showed a weak coagulant effect. The activity of the acetone-extract was due probably to heparin. In the subsidiary extraction the heparin was recovered almost quantitatively in the acetone-extract. The acetone extracts of the liver were dissolved in chloroform, filtered and precipitated with acetone. The supernatants were found inactive, but the precipitates were ½–⅛ as active as the parent materials. The Toluidin-blue test for heparin (Trethewie and Melvin, 1945) was markedly positive, and became weaker after every re-dissolving in chloroform and re-precipitating in acetone. The disappearance of activity as assessed in Quick's prothrombin test followed a similar course. The fractions from the alcoholic extractions at 4° C., 37° C., and from the "Soxhlet" extraction, showed moderate anticoagulant activity and negative Toluidin-blue tests. These fractions were dissolved in chloroform and precipitated in acetone repeatedly. The supernatants were inactive and the activity of the precipitates was considerably less than the activity pertaining to the parent material. The activity of the extracted tissue powder was quite negligible (equivalent to 1/30,000 mg./mg. heparin).

The only anticoagulant extract which could be concentrated further successfully was formed during the extraction with boiling absolute alcohol. The treatment of this fraction will now be described.

The extraction was carried out with three 1 litre vols. of absolute alcohol for 30 minutes each over a glycerol bath at 91° C. By means of an adaptor a flask was attached to a Davies

reflux condenser to the top of which was fitted a tube of silica-gel. The contents of the flask were filtered under suction through a Whatman No. 5 paper and the temperature of the mixture on the paper was kept at 73°–79° C. as the funnel was immersed in a glycerol bath at 82°–88° C. The three extracts were combined (2,700 ml.), filtered through a No. 1 Whatman paper at a temperature of 77°–80° C. The paper and funnel were washed three times with 50 ml. boiling absolute alcohol and the total 2,850 ml. alcoholic extract was kept at —14° C. for 48 hours, but no precipitate developed. The extract was concentrated, therefore, in a stream of N_2 to 500 ml. over a glycerol bath at 50° C., and 120 mm. pressure of mercury. Although no precipitate came down after 6 hours at room temperature and did not appear after a further 12 hours at 2½° C., a yellow fluffy precipitate formed after 48 hours at —14° C. When attempting to concentrate the precipitate by centrifugation the precipitate re-dissolved. Therefore, after another 36 hours at —14° C., the mixture was filtered on a fluted Whatman No. 5 paper at 2·5° C. The paper containing the precipitate was dried *in vacuo*, extracted with 200 ml. boiling chloroform and the extract kept for 6 hours at 2·5° C. A precipitate which formed in the presence of 1,400 ml. acetone, was kept for 12 hours at —14° C., and then centrifuged. This precipitate was labelled "A" and the supernatant was tested for anticoagulant activity by Quick's method, modified by Trethewie (1945a). It did not alter the clotting time. The filtrate (485 ml.) from the cold filtration was concentrated at 80 mm. Hg. pressure on a glycerol bath at 55° C. 28 ml. of syrup were obtained which on standing at —14° C. for 12 hours, formed a dark yellow fluffy precipitate, which was collected at 2·5° C. on a fluted No. 5 Whatman paper. This paper containing the precipitate was extracted with 60 ml. boiling chloroform, the extract allowed to stand for 6 hours at 2½° C., and then precipitated with 420 ml. acetone. This was kept at —14° C. for 12 hours and centrifuged. The material thus obtained was called "B" and the supernatant was devoid of anticoagulability. The filtrate from the second cold filtration contained no anticoagulant properties. "A" was a yellow crystalline powder, slowly changing its colour to orange-yellow in air. 222 mg. were collected and the anticoagulant activity was equivalent to 1/70 mg. heparin/mg. "B" which was originally a dark yellow crystalline material, on pulverising gradually became orange, and ultimately light brown, in the presence of air. 148 mg. were obtained and the activity was 1/120 equiv. heparin.

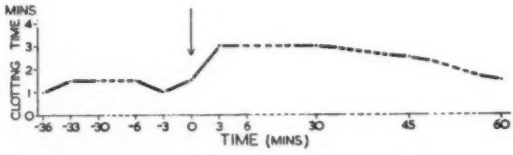

Fig. 1. Effect of extract E on clotting time.
Ordinate: clotting time (mins.).
Abscissa: time in minutes.
At the arrow 15 mg. extract injected intravenously.

128 mg. "B" were dissolved in 8 ml. chloroform, filtered, precipitated with 56 ml. acetone, allowed to stand at —14° C., for 36 hours and centrifuged. No anticoagulant activity could be detected in the supernatant. The precipitate "C" (99 mg.) was amber in colour and appeared as pale yellow crystals when pulverized. It changed slowly to a yellow colour when exposed to the air. The activity was 1/50 equiv. heparin. A similar method of concentration was carried out on 199 mg. "A" yielding 150 mg. "D" in respects to colour and anticoagulant activity like "C". Hence "C" and "D" were pooled and called "E".

Testing the anticoagulant effect of "E" in a rabbit: 17·9 mg. "E" were emulsified in sterile Ca-free Tyrode and 0·36 ml. of the emulsion containing 15 mg. "E" was injected into the marginal vein of a rabbit. The rabbit was bled from the other ear at various times after

the injection and the coagulation time was tested by the capillary method. The rabbit was bled before the injection for a comparison of the results.

The clotting time was estimated at intervals of 3 minutes. The results were 1′ 00″, 1′ 30″, 1′ 30″ in one experiment and 1′ 30″, 1′ 00″ and 1′ 30″ in another, when the animal was bled before the injection of the extract. The clotting time was 3′ 00″, 3 minutes, 3′ 00″ 30 minutes, 2′ 25″, 45 minutes and 1′ 30″, 60 minutes, after the injection of 15 mg. "E" (Fig. 1). There were no untoward effects noted in the rabbit.

200 mg. "E" were dissolved in 25 ml. 1:5 absolute alcohol-ligroin mixture, filtered, precipitated with 25 ml. absolute alcohol, allowed to stand at —14° C. for 3 days and centrifuged. This procedure was repeated twice and the three supernatants united, dried *in vacuo* over a glycerol bath at 58° C., in a stream of N_2. The residue was a sticky yellowish-brown material, weighing 62·6 mg. (F). The final precipitate "G" was similar to its parent material, weighing 21·1 mg. and having 1/80 equiv. heparin activity.

Filter-paper-partition chromatography.

10 mg. "E" were dissolved in 5 ml. chloroform and this solution was used as stock in an attempt to achieve the separation of the active principle by filter-paper chromatography. The method of Rockland and Dunn (1949) was applied in the first instance, using strips of Whatman No. 1 paper. The filter paper was purified before use by passing distilled water through it (Wynn, 1948) and drying to a constant weight at 91° C. Except in the first instance when colloidine, water and ethanol were used as the solvent, the filter paper, after being purified, was rendered unwettable with water by the following treatment (Trikojus and Stanley, 1948): The air-dried paper was exposed in a closed vessel to vapours of $(CH_3)_2$ $SiCl_2$ for 5 minutes, then to NH_3 for 3 minutes and finally washed thoroughly with acetone and dried at 28° C. After the solvent front came within 3 mm. of the end of the strip, the strips were removed and dried. Each then was inspected under ultra-violet light and the fluorescent areas marked. The bluish-white fluorescent spots were distinguished easily, as the untreated paper showed no noticeable fluorescence and the waterproofed paper, a faint purplish-pink shade. The fluorescent and non-fluorescent areas were cut out and separately extracted for 20 minutes under reflux with boiling chloroform and the chloroform was then evaporated *in vacuo* at 50°–55° C. The residue was weighed and tested for anticoagulant activity. Various solvent mixtures were employed including colloidine, water and ethanol, various ratios of water, ethanol and chloroform, and a mixture of ligroin, absolute alcohol and water.

(1) Colloidine, saturated with water, containing 13 p.c. ethanol: Two fluorescent areas were visible, one at the point of application and a much stronger one at the solvent front. The extracts of both fluorescent areas showed anticoagulant activity, but the non-fluorescent area, in the intermediate part of the strip, constituting $\frac{1}{3}$ area of the strip, showed no activity.

(2) Various ratios of water, ethanol and chloroform. The fluorescent spots were dispersed throughout the strip. No difference in the anticoagulant activity could be detected between the extracts of the different areas of the strips using the various proportions of constituents of the solvent.

(3) 50 p.c. ligroin, 43 p.c. absolute alcohol and 7 p.c. water: Results as in (1).

These runs were considered exploratory as the quantity of "E" used in each was 52 μg. (13 drops of 2 μl. stock solution). It was equivalent to 1 μg. heparin in anticoagulant activity (near to the experimental error of the estimation of anticoagulant activity by the method used).

Run 1, using colloidine, saturated with water containing 13 p.c. ethanol showed the most promise and was repeated on a larger scale, by the method of Consden, Gordon and Martin

(1944). Four washed sheets of No. 1 Whatman paper were used and 1 mg. "E" applied (at 25 equidistant points along the starting line using 10 drops of 2 μl. each) to each sheet and the corresponding areas of each sheet were united for extraction. From each of the two fluorescent areas (the starting line and solvent front) approximately 1/3 of the original anticoagulant effect could be recovered; as these areas were 1/17 and 1/10 respectively of the total area of the paper, the partition effect was reasonably good. No purification, however, was obtained by this experiment. From both the fluorescent and nonfluorescent areas of the paper, a considerable quantity (16 × original weight) of a sticky, brown material was extracted. No investigation concerning this material has been carried out, but the fact that the material was obtained by chloroform extraction of the filter paper, after the paper was purified, together with the appearance of the material, suggests that it differs from the water-soluble impurities found in the same type of paper by Wynn (1949).

Further attempt at extraction.

Another series of experiments was performed to obtain a greater amount of a substance corresponding to "E". The livers of four dogs were obtained as before. 930 gm. tissue paste were spun-frozen at —20° C., freeze-dried at —20° C. *in vacuo* for three days and pulverized in a mill. An attempt was made to shorten the acetone, ether and alcohol extractions of the non-anticoagulant materials, prior to the hot alcohol extraction of a material analogous to "E". The tissue powder (359 gm.) was mixed thoroughly with 54 gm. of powdered porous glass and treated under reflux with 2,200 ml. boiling absolute acetone, three times for 30 minutes; dried and treated under reflux for 30 minutes with three batches of 2,100 ml. boiling absolute ether and dried. The material then was treated with 2,300 ml. absolute alcohol for three periods of 24 hours. All extracts were prepared in solid form by removing the solvents *in vacuo*. Neither the ether-extract nor the warm alcohol-extract had any anticoagulant activity; the acetone-extract had considerable activity, attributable to heparin. This anticoagulant activity could not be recovered from the precipitate or supernatant, when the extract was dissolved in chloroform and precipitated with acetone. The disapperance of the anticoagulant effect was accompanied by a positive-negative change in the Toluidin-blue tests.

Up to this point, the experiment proceeded as expected on the grounds of the previous work. From the boiling alcoholic extractions however, carried out with the same technique as before, a voluminous, white precipitate settled on cooling. When separated by filtration and dried *in vacuo*, a greyish-white, brittle layer formed on the filter paper. It could be removed easily and turned dark grey in the air. 2·223 gm. with 1/370 equiv. heparin activity were obtained. This material was treated further by dissolving in chloroform, filtering and precipitating with several times its own volume of acetone. This was repeated four times and the final precipitate was dissolved in 5:1 ligroin-absolute alcohol. This was precipitated with an equal volume of absolute alcohol. This was repeated five times. The nine supernatants and the nine filter-residues were united, dried *in vacuo* and found to be inactive. The final precipitate weighed 200·4 mg. and was a brittle, greyish-brown material with an anticoagulant activity of 1/1,000 equiv. heparin.

The tissue powder dried after the hot alcoholic extraction, weighed 342 gm. and had an anticoagulant activity equivalent to 1/200 mg./mg. heparin. This powder was extracted under reflux with 1,200 ml. boiling 3:1 chloroform-methanol mixture for three periods of 30 minutes. The anticoagulant activity of this extract was negligible and consequently it did not warrant further treatment. The tissue powder at the corresponding stage in the previous experiment was practically free of activity (1/30,000 equiv. heparin) whereas this powder still possessed 1/300 equiv. heparin.

The powder was allowed to stand for 48 hours twice at 37° C. with 1,500 ml. Ca-free Tyrode containing 3 ml. toluene. Then it was collected on a Buchner, dried and pulverized.

The weight of the powder, which possessed no anticoagulant activity was 310 gm. The Tyrode-extract was concentrated *in vacuo* to 1/10 its volume over a water bath at 40° C. The residue was freeze-dried. 96 mg. of grey, foamy, brittle material was obtained and it was ground up and extracted in a "Soxhlet" apparatus with 250 ml. chloroform. The resultant residue was a sticky black gum, weighing 263 mg. and possessing no anticoagulant activity. The chloroform-extract was concentrated *in vacuo* to 30 ml. and 210 ml. acetone were added. The supernatant was free of activity. The precipitate consisted of yellow flakes, weighing 4·6 mg. and possessing no activity.

DISCUSSION.

Chargaff (1937) extracted an anti-coagulant from normal mammalian tissues, and a similar one (Chargaff 1938) from the spleen in a case of "Niemann Pick" disease. This substance accompanied sphingomyelin throughout the purification, up to ligroin-alcohol precipitation, when a purified, non-anti-coagulant sphingomyelin remained in solution and the anti-coagulant activity was found in the precipitate which contained impure sphingomyelin. He did not purify the anti-coagulant further, but showed that this fraction did not contain carbohydrates: therefore it was not heparin. However, it contained sulphur, thus it is either not identical with the sulphur-free anti-coagulant of De Sütö-Nagy (1944) or the sulphur or its compound is an impurity.

It has been shown (Trethewie, 1945b; Trethewie, Cleland and Pengelley, 1947; and Trethewie and Day, 1949) that an additional anti-coagulant property was conferred on diluted heparinized blood when perfused through the liver and other organs. It appeared from the above work that this anti-coagulant, which was liberated under near-physiological conditions, might be related to the anti-coagulant referred to above. Insufficient anti-coagulant was obtained by this method for a larger-scale purification. We therefore extracted mammalian liver in an attempt to purify and test the anti-coagulant for lipoid-solubility.

We were able to obtain about a quarter of a gram of a lipoid-like substance, having approximately 1/50 activity of heparin. This contained sulphur and phosphorus; its anti-coagulant activity was clearly not due to heparin because the treatment by which it was extracted from the liver-paste, and the subsequent steps in purification were unsuitable for any but insignificant traces of heparin to reach this stage of preparation. The high solubility of heparin in acetone would in itself be a sufficient guarantee for the correctness of this claim, but for further corroboration, 275 mg. heparin were mixed with the extracted tissue-powder, and this mixture subjected to extractions corresponding to those by which the anticoagulant substance was obtained from the liver-paste. An activity, equivalent to approximately 85 p.c. of the heparin added, could be found in the acetone-extract, but the fraction, (corresponding to the active, lipoid-like-substance gained from the original liver-paste) was either inactive or possessed an insignificant slight coagulant-activity.

The anti-coagulant, lipoid-like substance is of a pale yellow colour; this colour might possibly be due to inactive impurities, as successive purifications (which increased, or, at least, left intact the anti-coagulant activity) caused it to fade gradually; exposure to air deepened this colour to a bright yellow (in case of impure samples to a dark orange). The substance is very highly soluble in hot chloroform, freely soluble in cold chloroform, hot alcohol, hot methanol, insoluble in acetone, and water. It does not emulsify in distilled water, but emulsifies very easily in weak solutions of electrolytes, particularly of salts and alkalis.

We further investigated this anti-coagulant by paper partition-chromatography and found that in partitioning between colloidine and water, the anti-coagulant-containing portions of the paper were fluorescent under U.V. light. The rate of concentration of anti-coagulant by a single operation was approximately sixfold, indicating approximately 1/9 of the activity of heparin. However, owing to the presence of a chloroform-soluble impurity in the water-purified filter-paper, the substance recovered from the paper was considerably more impure than the substance applied to the paper.

As the two fluorescent areas (containing active material) were rather far removed from each other (one being just below the origin, the other one on the solvent front), it seems likely that either at least two, chemically distinct, different anti-coagulant substances are present in the mixture we attempted to purify and concentrate, or some of the active principle for some reason attached itself at the site of application.

We consider it rather difficult to account for the failure to repeat the extraction of an anti-coagulant substance, (analogous to the one obtained from the livers of the first three dogs), from a subsequent batch of four dog-livers. There seems to be no doubt that a large quantity of anti-coagulant, other than heparin, was present in these four livers, as, after the unsuccessful solvent-extractions, the residue-powder still contained activity equivalent to approximately *one gram* heparin. The obvious explanation would be to assume that the anti-coagulant was adsorbed to the proteins so intensely that the solvents could not remove it; this, however, is rather unlikely, as boiling polar solvents are known to denature proteins, and liberate lipoids from them. Further, we have attempted to take up the anti-coagulant present in the extracted powder by Tyrode-solution; this attempt was encouraged by the high anti-coagulant activity shown by this powder when a small sample was treated with Tyrode for testing; but the bulk Tyrode-extract when freeze-dried and chloroform-extracted did not show anti-coagulant activity. These results are made more paradoxical by the absence of anti-coagulant activity in the residue from Tyrode extraction. We are rather unwilling to credit the freeze-drying of the liver-paste, (prior to extraction) with being the cause of these unexpected discrepan-

cies. It may be that the livers or one of them in the second batch contained an abnormal substance.

It is considered important to identify and purify this anti-coagulant since it is a "physiological" one, being readily found in normal tissue, and there are certain disadvantages attaching to the two main ones at present in use. Heparin must be given intravenously (or more recently intramuscularly). Its action is also not very prolonged. Coumarin compounds may be given orally but may have severe delayed effects.

While we have not yet obtained our anti-coagulant in a high degree of purity, from its solubility in fat solvents it may be possible to use it as vitamin K has been, namely dissolved in oil and inuncted. This would combine ease of application with more prolonged action.

Following the injection of 15 mg. intravenously in the rabbit the blood clotting time was increased and the normal was not reached till one hour had passed. The significance of the increase of clotting time was proven statistically: "Student" t-test yields $\cdot 001 < P < \cdot 01$: a highly significant result. This is equivalent (anti-thromboplastin activity) in dose to 0·3 mg. heparin (approx.). The effect of twenty-five times this dose of heparin is largely over in 4 to 5 hours.

Our anti-coagulant is relatively stable. It has remained active in solution in the cold room for over a period of weeks while the heparin content of the solution has degraded. It will withstand boiling with fat solvents and acetone. No untoward effects were noted in the rabbit (above) following the injection of the anti-coagulant though histological examination and examination for delayed effects were not carried out. These will be done when larger amounts are available and preferably in a purer state.

We have been able to show that anti-coagulant is liberated under the influence of a slow rate of flow only in blood-perfused organs. We suggest that this anti-coagulant owing to its solubility and general appearance is similar to the one we extracted directly from the tissues but we have not purified the substance sufficiently to prove this point as yet.

SUMMARY.

Details are recorded of the extraction and partial purification of an anticoagulant obtained from mammalian liver.

A pale yellow substance soluble in alcohol with 1/50 the activity (approx.) of heparin has been obtained.

Partition chromatography yielded further concentration of the degree of activity.

The active area in chromatography is fluorescent to ultra-violet light.

The intravenous injection of 15 mg. into the rabbit reduces the coagulability of the blood for one hour with a high degree of significance.

This publication describes orientation tests to indicate possibly effective and efficient avenues of purification. It is hoped that the study of this substance will be continued in the near future.

Acknowledgments. Our thanks are due to Professor V. M. Trikojus and Professor R. D. Wright (University of Melbourne) and Drs. I. W. Wark and F. G. Lennox (Division of Industrial Chemistry, C.S.I.R.O.) for their generous help and kind permission to carry out some of the experiments in their departments; to Mr. H. R. Marston (Division of Biochemistry, and General Nutrition, C.S.I.R.O.) and Professor L. G. H. Huxley (Adelaide University) for ready help with equipment. Thanks are also due to Drs. A. Greenhill and H. Lindley, Mr. P. Stanley, Miss W. Williams, and Dr. V. Wynn, (C.S.I.R.O., Melbourne, and the University of Melbourne) for help and advice.

REFERENCES.

Carter, H. E., Haines, W. J., Ledyard, W. E. and Norris, W. B. (1947): J. biol. Chem., 169. p. 77.

Chargaff, E. (1937): *Ibid.*, 121, p. 175.

Chargaff, E. (1938): *Ibid.*, 125, p. 677.

Consden, R., Gordon, A. H. and Martin, A. J. P. (1944): Biochem. J., 38, p. 224.

Consden, R., Gordon, A. H. and Martin, A. J. P. (1946): *Ibid.*, 40, p. 33.

Erlansden, E. (1907): Z. physiol. Chem., 51, p. 71.

Quick, A. J. (1942): The Hemorrhagic Diseases and the Physiology of Hemostasis. Springfield, Thomas.

Rockland, L. B. and Dunn, H. S. (1949): Science, 109, p. 539.

de Sütö-Nagy, G. J. (1944): J. biol. Chem., 156, p. 433.

Thannhauser, S. J., Benotti, J. and Boncoddo, N. F. (1946): *Ibid.*, 166, p. 669.

Trethewie, E. R. (1945a): Austral. J. exp. Biol., 23, p. 103.

Trethewie, E. R. (1945b): Med. J. Austral., II, p. 328.

Trethewie, E. R., Cleland, J. B. and Pengelley, T. J. (1947): *Ibid.*, 1, p. 321.

Trethewie, E. R. and Melvin, P. A. (1945): Austral. J. exp. Biol., 25, p. 241.

Trethewie, E. R. and Day, Allan J. (1949): Med. J. Austral., 11, p. 867.

Trikojus, V. M. and Stanley, P. A. (1949): Personal communication.

Thierfelder, Klenk, Schuhwirt, Walz *et al.* (1927): Z. physiol. Chem., 166, p. 210. *Ibid.* (1927), 166, p. 269. *Ibid.* (1930), 193, p. 59. *Ibid.* (1933), 218, p. 1. *Ibid.* (1933), 218, p. 153. *Ibid.* (1933), 221, p. 27. *Ibid.* (1933), 221, p. 259. *Ibid.* (1934), 229, p. 151. *Ibid.* (1940), 262, p. 128. *Ibid.* (1941), 267, p. 128. *Ibid.* (1942), 273, p. 76.

Wynn, V. (1948): Personal communication.

Wynn, V. (1949): Personal communication.

ADDENDUM[2]

For the extraction of the anticoagulant described earlier in this paper, we felt the need of equipment suitable for the vacuum distillation of solvents from extracts with the subsequent vacuum drying of the residue so that it could be handled in small quantity. We also wished to obtain the residue in a form in which it could be stored easily and it was particularly important that it should be free from both rubber and tapgrease; it possesses a negative surface tension.

The equipment (Fig. 2) consists of a pyrex test tube, 10½ inches long and 1⅞ inches inside diameter, (4) fitted with a female quickfit B40 joint, and a quickfit standard adaptor DA26 (3) (male B40 and female B19) fitting into this test tube. Between the two ground parts of the adaptor, four glass hooks are fitted with inner seals; from these hooks a loop (made of silver wire) can be safely suspended with the help of four silver hooks. Several loops of different widths and lengths can be suspended inside the large test tube. The inner test tube is charged with the extract solution which is to be dried.

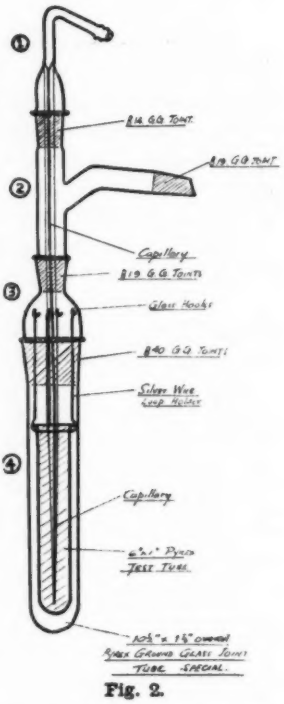

Fig. 2.

Into the DA26 adaptor, a quickfit standard adaptor SH4/24 (2) is fitted; male B19 joint is connected with female B14 joint and, at an angle, to another male B19 joint. The latter connects with a suitable Liebig-condenser and the former carries the capillary, (1). A capillary of suitable length is joined with the inner seal to the top of a hollow, male quickfit B14 joint.

Acknowledgment. Our thanks are due to Mr. B. C. Fuller of the Institute, and to Laboratory Supplies Ltd., for technical assistance.

[2] By F. E. Binet, E. R. Trethewie and G. A. Gormly.

ADENOSINE TRIPHOSPHATE AND THE ADRENALIN-LIBERATING ACTION OF SERUM

by G. REID[1]

(From the Department of Physiology, University of Melbourne).

(Accepted for publication 7th March, 1951.)

It is well known that adenosine triphosphate (A.T.P.) causes contraction of smooth mammalian muscle (Buchthal and Kahlson, 1944a) and of striated muscle of both mammal and frog (Buchthal and Folkow, 1944; Buchthal and Kahlson, 1944b, 1946). Adenosine triphosphate also stimulates the perfused superior cervical ganglion of the cat (Lundberg, 1947; Feldberg and Hebb, 1948).

Cat serum is known to liberate adrenaline from the suprarenal gland of the cat, an action which is not shared by adenosine or adenylic acid (Reid, 1948, 1951). Feldberg (1948) having in mind the action of A.T.P. on the anatomically analogous superior cervical ganglion, suggested in a personal communication that this substance may be responsible for the action on the suprarenal gland. Adenylic acid has been isolated from fresh serum or defibrinated blood by Zipf (1931) but he points out that his methods did not exclude the possibility of the presence of A.T.P.

This communication reports the action of A.T.P. on the suprarenal medulla of the cat and disposes of the possibility that this is the substance in cat serum which liberates adrenaline.

METHODS.

Two samples of the barium salt of A.T.P. were used. One was kindly given by Professor Ennor of the National University; the other was Adenosine Triphosphate, Bischoff (Ba_2 ATP, $4H_2O$), "assayed by transphosphorylating the labile P to glucose in the presence of hexokinase and myokinase and measuring the adenylic acid formed by enzymatic deamination in the spectrophotometer." Assayed in this way its purity in terms of labile phosphorus is stated to be 101·6 p.c. Solutions of sodium A.T.P. were prepared by dissolving the barium salt in ice-cold N/10 hydrochloric acid, precipitating most of the barium by the addition of a solution of sodium sulphate, neutralizing with N/10 sodium hydroxide and precipitating the rest of the barium as sulphate using rhodazonic acid as an external indicator.

The action of A.T.P. on the suprarenal gland of the cat was tested by the method of Feldberg and Minz (1931), the animal being anaesthetised with chloralose after a preliminary ether anaesthetic, the vagi divided, artificial ventilation set up and evisceration carried out.

1 Technical assistance was provided by a grant from the National Health and Medical Research Council.

The aorta and the left or both renal arteries were tied and injections were made into the cut stump of the superior mesenteric artery.

The pressure in the left common carotid artery, and, in some experiments, the contraction of the right nictitating membrane which had been denervated some 10 or more days previously were recorded.

The adenyl content of cat serum was estimated by injecting 1 ml. or less of the serum or a filtrate prepared from it, into the left auricle of the guinea-pig and recording the contraction of the right auricle (Drury, Lutwak-Mann and Solandt, 1938).

RESULTS.

The action of A.T.P. on the guinea-pig auricle.

There does not appear to be any statement in the literature as to whether A.T.P. acts on the guinea-pig auricle in a manner similar to adenosine or adenylic acid and can be estimated as an adenyl compound by the method of Drury, Lutwak-Mann and Solandt (1938). These workers do not refer to A.T.P. in their paper. In a footnote to the paper of Drury and Szent-Gyorgy (1929) it is implied that the action of adenylic acid in causing heartblock in the guinea-pig is shared also by adenyl pyrophosphate. On the other hand Robertson and Shaw (1941) find that its action on the isolated frog's heart is different from that of other adenyl compounds.

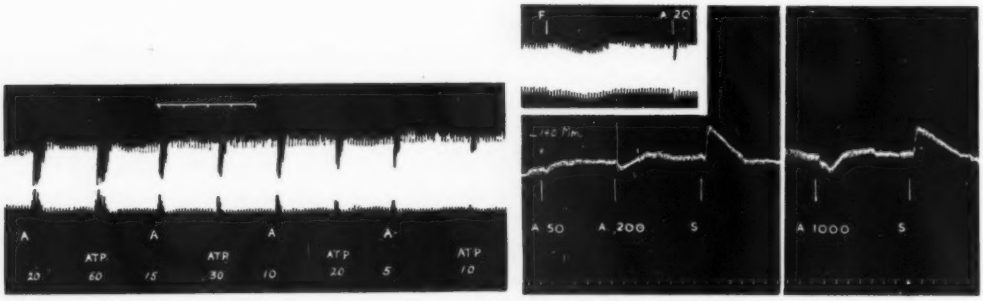

Fig. 1. Record of right auricular contractions of an atropinized guinea-pig. At A.T.P. adenosine triphosphate and at A adenosine were injected into the left auricle, the figures referring to microgrammes. Time in half minutes.

Fig. 2. Arterial blood pressure of an eviscerated cat prepared as described in text. Adenosine triphosphate was injected into the cut stump of the superior mesenteric artery at A, and 0·5 ml. of cat serum at S. The inset shows the action of a filtrate corresponding to 0·5 ml. of the same cat serum at F, and 20 μg. of A.T.P. at A, on the guinea-pig auricle. Time in half minutes.

Fig. 1 shows the response of the guinea-pig auricle to a series of injections alternately of adenosine (BDH) and A.T.P. It illustrates that A.T.P. does, in fact, cause a similar response of the guinea-pig auricle to adenosine but that the dose required for a quantitatively similar response is about three times as great (expressed in terms of the Ba salt). If the dose of A.T.P. is expressed in terms of its adenosine content its action will be approximately equivalent to the same dose of adenosine.

Serum does not mask the action of A.T.P. for when added to serum prior to injection it gives the expected response.

The adenyl content of cat serum.

Blood was withdrawn from the carotid artery of anaesthetized cats, allowed to clot and the serum removed by centrifugation. Such serum: (1) was kept at room temperature for 0·75 to 2 hours from the time the blood was taken and injected into the vascular supply of the suprarenal of the same cat which had been prepared in the meantime, or (2) was placed in the refrigerator and injected into another cat some 24 hours later. In the former event, at the same time as an injection was made into the cat a portion of the serum was boiled with an equal volume of saline to stop enzyme activity, filtered and the filtrate subsequently tested for adenyl compounds. In the latter event the serum itself was directly tested on the guinea-pig auricle some hours before injection into the cat.

Although Zipf (1931) was able to isolate large amounts of adenylic acid from fresh defibrinated blood of several species (several mg./100 ml.) many samples of cat serum which stimulated the adrenal medulla contained no detectable adenyl compound (less than 2 μg. per ml.). This is doubtless due, in part at least, to a deaminating enzyme in cat serum (Drury *et al.*, 1938), but even the freshest serum contained less than 10 μg. per ml.

The action of A.T.P. on the suprarenal medulla.

When A.T.P. in doses ranging from 10 μg. to 5 mg. (as Ba salt) is injected into the cut stump of the superior mesenteric artery, neither the blood pressure record nor the record of the nictitating membrane indicates an early and abrupt liberation of adrenaline such as occurs with acetyl choline, potassium ions or with serum itself. With doses up to about 0·2 mg. there was little or no change in the records. Fig. 2 shows the responses to 50, 200 and 1,000 μg. of A.T.P. at A and of 0·5 ml of cat serum at S each injection being given into the cut stump of the mesenteric artery. When the first injection of serum was given a sample was boiled and the filtrate so obtained was tested for adenyl compounds. The inset in the Figure shows the response of the guinea-pig auricle to this filtrate representing 0·5 ml. of serum at F and to 20 μg. of A.T.P. at A. Clearly, A.T.P. could not be responsible for the adrenaline-liberating action of this sample of serum. With doses greater than about 0·2 mg. there is an initial fall of blood pressure, the depression lasting 1 to 4 minutes. Sometimes the fall of blood pressure is succeeded by a rise to a level somewhat higher than initially followed by a rather slow decline. When the nictitating membrane is recorded also, it is frequently found to relax during the period of depression especially when its tone is high, followed by a slight contraction during the subsequent rise indicating that adrenaline is liberated during this phase, although a direct action on the smooth muscle cannot be excluded. In any case such adrenaline liberation is not due entirely to a direct action of A.T.P. in the gland itself for similar responses in both blood pressure record and nictitating membrane could be seen when the drug was given intravenously. It can be explained simply as a secondary result of the initial fall of blood pressure or as a central or reflex effect of the A.T.P. itself (cf. Emmelin and Feldberg, 1948). Even experiments in which the suprarenals were denervated and A.T.P. injected intra-arterially gave no clear indication of a direct action in the gland, for the secondary rise of blood pressure, which in any case was small, could be explained by a reflex vasoconstriction and the slight response of the nictitating membrane as due to a direct action of the A.T.P. on the smooth muscle. The liberation of adrenaline may be very pronounced when as a result of intravenous injection of A.T.P. the fall of blood pressure is great and is prolonged for some minutes, the pulsations disappearing from the mercury manometer. When recovery occurs from such a profound depression the blood pressure rises rapidly, the heart rate increases, and the nictitating membrane gives almost a maximal contraction. If the thorax be opened during the phase of depression the left auricle appears almost empty and the right auricle and pulmonary artery are engorged with blood. This is in conformity with the observations of Emmelin and Feldburg (1948) who described pulmonary vasoconstriction following the intravenous injection of A.T.P.

In four experiments I have examined the possibility that A.T.P. may potentiate the action of acetylcholine on the adrenal medulla (on analogy with its effect in skeletal muscle). No potentiation occurred when acetylcholine was given simultaneously or up to five minutes after the administration of A.T.P. in atropinised or unatropinised animals.

DISCUSSION.

In the first place, these experiments show that the action of A.T.P. on the guinea-pig auricle is similar to that of other active adenyl compounds and therefore serum which fails to inhibit this test object can be considered free not only from adenosine or adenylic acid but also from A.T.P. in significant quantities. In fact the activity of adenosine does not appear to be significantly altered quantitatively by the addition of the phosphate groups to the molecule.

Secondly, the experiments make it clear that A.T.P. cannot be responsible for the adrenaline-liberating action of serum for active serum may be devoid of adenyl compounds, whereas A.T.P. in doses up to 5 mg. does not possess an action similar to serum. Moreover, we have more recently shown (Rand and Reid, 1951), that the vasoconstrictor substance of serum isolated by the method of Rapport *et al.* (1948) also stimulates the suprarenal medulla.

In view of the stimulating action of A.T.P. on skeletal and smooth muscle and on the superior cervical ganglion it is surprising that no clear-cut effect on the suprarenal medulla was recorded. One possible explanation may be that A.T.P. does in fact stimulate the gland but that the dosage used here has been inadequate. When one compares them with those used by Feldberg and Hebb (1948) (who expressed theirs in terms of the labile P) it is found that the maximum doses used are of the same order but these workers injected the drug into a perfusion cannula supplying the superior cervical ganglion at a rate of about 0·2 ml./min. The rate of flow through the suprarenal gland is probably much greater than this (Vogt, 1943), and in some experiments in which the right renal artery was left unligated much of the injected dose probably by-passed the suprarenals. Hence it is likely that the concentration of A.T.P. reached in the vessels of the gland is less than the corresponding figure reached by others in the superior cervical ganglion. With the largest doses, however, the vascular effects of the A.T.P. are likely to mask those of liberated adrenaline and even the nictitating membrane may relax initially. In short, the smaller doses of A.T.P. do not stimulate the gland while the larger doses produce effects not confined to the suprarenal which interfere with interpretation. It is proposed to explore this aspect further, using an isolated perfused gland.

SUMMARY.

The action of A.T.P. on the guinea-pig auricle is indistinguishable from that of adenosine or adenylic acid.

A.T.P. is not responsible for the stimulatory action of cat serum on the adrenal medulla.

REFERENCES.

Buchthal, F. and Folkow, B. (1944): Acta Physiol. Scand., 8, p. 312.
Buchthal, F. and Kahlson, G. (1944a): *Ibid.*, 8, p. 325.
Buchthal, F. and Kahlson, G. (1944b): *Ibid.*, 8, p. 317.
Buchthal, F. and Kahlson, G. (1946): *Ibid.*, 11, p. 284.
Drury, A. N., Lutwak-Mann, C. and Solandt, O. M. (1938): Quart. J. exp. Physiol., 27, p. 215.
Drury, A. N. and Szent-Gyorgi (1929): J. Physiol., 68, p. 213.
Emmelin, N. and Feldberg, W. (1948): Brit. J. Pharmacol., 3, p. 273.
Feldberg, W. and Hebb, C. (1948): J. Physiol., 107, p. 210.
Feldberg, W. and Minz, B. (1931): Arch. exp. Path. Pharm., 86, p. 266.
Lundberg, A. (1947): Acta Physiol. Scand., 13, p. 277.
Rand, M. and Reid, G. (1951): Unpublished observations.
Rapport, M. M., Green, A. A. and Page, I. H. (1948): J. biol. Chem., 176, p. 1,243.
Reid, G. (1948): Austral. J. exp. Biol., 26, p. 107.
Reid, G. (1951): *Ibid.*, 29, p. 101.
Robertson, I. and Shaw, F. H. (1941): *Ibid.*, 19, p. 207.
Vogt, M. (1943): J. Physiol., 102, p. 341.
Zipf, K. (1931): Arch. exp. Path. Pharm., 160, p. 579.